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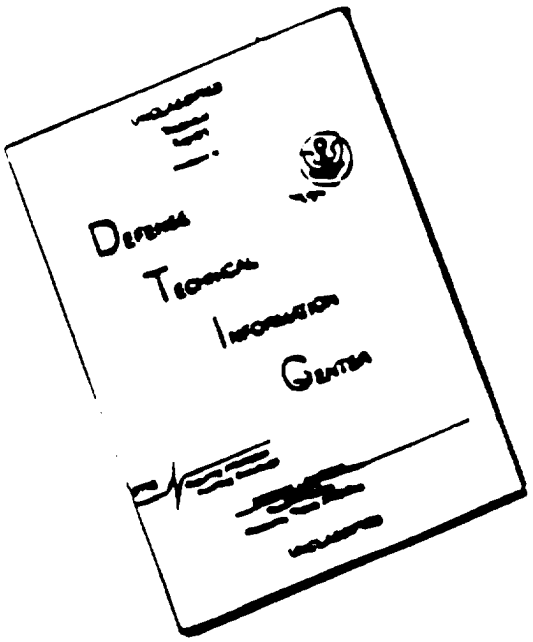

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Mutation of the p53 gene is very frequent in breast cancer. In normal cells induction of wild-type p53 function leads to either cell cycle arrest or cell death. Loss of this function can contribute to oncogenic cell transformation. Additionally the presence of mutant forms of p53 in breast tumor cells may actually facilitate the process of tumorigenesis. The properties of mutant p53 proteins in vitro and in breast tumor cell lines will be studied, experiments will focus on analysis of the structure and modification of mutant p53 proteins as well as the effect of cellular signalling on p53 function. Additionally it is planned to establish breast cell lines expressing inducible mutant p53 to determine the effect of such mutants on parameters of cell cycle, growth and death.

We have discovered that all mutant forms of p53 tested are capable of binding specifically to p53 response elements present in p53 target genes at lower but not at physiological temperatures. Furthermore, we have identified a means by which such binding is stabilized at the higher temperature. This will allow us to explore means to develop molecules that might have the outcome of converting p53 in breast tumor cells from mutant to wild-type in function. One approach will be to develop a yeast-based screen for mutant p53 modifying genes. Such reagents would have clear therapeutic advantages.

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Carol Reeves Aug 14/96

PI - Signature Date

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DAMD-17-94-J-4275

The goal of the research funded by DAMD-17-94-J-4275 is to characterize the role of mutant p53 in breast cancer progression and to develop means to counteract the tumorigenic potential of mutant p53.

INTRODUCTION:

The p53 tumor suppressor protein plays a pivotal role in transmitting a signal from agents that induce genotoxic stress to genes that control the cell-cycle and apoptosis (1,2). p53 is a DNA binding dependent transcriptional activator which binds specifically to sites in genomic DNA that contain two or more copies of the consensus sequence: 5' R R R C A/T T/A G Y Y Y 3' (3). Such sites are identified as p53 response elements in a number of genes. Thus when cells are stressed by processes such as DNA damage or hypoxia the p53 protein normally present in low quantities in cells and in a latent, inert form, is activated both quantitatively and qualitatively to induce several target genes. Among these are included the genes expressing GADD45 (4), WAF1/p21/CIP1 (5), mdm2(6), cyclin G (7), bax (8) and IGFBP3 (9). Each of these genes contains a p53 response element and is therefore a likely target for p53 as a transcriptional activator to induce their expression. Each thus is likely to play a role in the p53 pathway in which, as a result of DNA damage, normal cells either undergo cell cycle arrest or cell death. When p53 is mutated and cannot respond thus to DNA damage, cells display the loss of growth control that is characteristic of tumorigenesis.

Mutation of the p53 tumor suppressor gene is among the most frequent events in breast cancer. Such mutation is frequently manifested as loss of one allele coupled with missense mutation of the other allele. Strikingly the location of the missense mutations are within the central region of the molecule (10) and this region contains the DNA binding domain (11). This highlights the likelihood that specific DNA binding and sequence specific transactivation is essential for the tumor suppressor function of p53 and that DNA binding is absent from mutant forms of p53. The missense mutant p53 proteins are frequently expressed at very high levels in breast tumor cells (12), and the p53 status in breast cancer has been linked closely to detection of p53 protein by immunostaining (13). Therefore understanding the function of the wild-type p53 protein and how it is altered when p53 is mutated will be critical to evaluating the prognosis of breast cancer. Importantly, the study of the properties of mutant p53 in breast cancer will hopefully lead to the development of ways to convert mutant protein to wild-type in function. The original aims of this proposal are as follows:

Specific Aims:

(1) Analysis of the structural properties of mutant forms of p53 that are found in breast cancer and how they differ from that of the wild-type form as well as among themselves.

(2) Analysis of the DNA binding properties of mutant forms of p53 in breast cell lines with the aim of (a) finding cellular genes that are targets of mutant p53 activation and (b) conversion to or stabilization of the wild-type DNA binding activity of mutant forms of p53.

(3) Identification of cellular proteins from mammary cell lines that might be involved in mutant p53 gain-of-function in breast cancer.

BODY

Stabilization of mutant p53 temperature sensitive DNA binding by N-terminal specific antibodies

The missense p53 mutations that occur with high frequency in human cancers are located within the central region of the protein that spans the sequence specific DNA binding domain. We examined in detail the DNA binding properties of four tumor derived mutant p53 proteins immunopurified from baculovirus infected cells. The mutations, val143□ala, arg175□his, arg248□trp, arg 249□ser and arg273□his are located within each of the four central conserved regions and the latter four are "hot-spot" mutations. We observed that while all mutants are defective for binding to DNA at 37□C each can bind specifically to several cognate p53 binding sites at sub-physiological temperatures (25-33□C). Furthermore, val143□ala, arg248□trp and arg273□his are capable of activating transcription from a p53-responsive promoter in cells at 26□C. Heating mutant p53 proteins at 37□C irreversibly destroyed their ability to subsequently bind at 25□C. However we found that several different monoclonal antibodies that each share the ability to recognize an epitope encompassing amino acids 46-55 markedly stabilized binding by mutant p53 proteins at 37□C. Both intact antibody and FAb fragments stabilized mutant p53 DNA binding. By contrast, antibodies that recognize epitopes located elsewhere within p53 stabilized mutant p53 binding significantly less effectively.

The results of these experiments have been recently published:
Friedlander, P., LeGros, Y. Soussi, T. and Prives, C. 1996. Regulation of mutant p53 temperature sensitive DNA binding. J. Biol. Chem. 271: p25468 (1996)

A mutant p53 that discriminates between p53 responsive genes cannot induce apoptosis

We have performed a study in collaboration with M. Oren and Y. Haupt (Weizmann Institute) examining the ability of wild-type and tumor derived mutant forms of p53 to activate transcription and induce apoptosis in transiently transfected p53 null H1299 cells using a procedure developed in the Oren laboratory (14). Having shown that several p53 mutants were capable of transcriptional activation in cells at 26□C we then examined the ability of p53 constructs to activate transcription at 32□C. In this case only one mutant form of p53 (ala143) was capable of transcriptional activation of a reporter containing the p53 responsive mdm2 promoter. This observation led us to examine the ability of p53 (ala143) to activate a number of other wild-type p53 responsive promoters. Unexpectedly, we found that while p53 (ala143) displayed comparable or greater levels of activation when

compared to wild-type p53 with reporters containing GADD45, p21 and cyclin G promoter elements, the mutant p53 was incapable of activating from the p53 responsive portions of the *Bax* and IGF-BP3 (not shown) promoters. Since both bax (8) and IGFBP3 (9) gene products have been shown to be involved in the cell death response we then tested the ability of mutant forms of p53 to induce apoptosis at 32□C. We found that while human wild type (wt) p53 can induce apoptosis in transiently transfected H1299 cells maintained at 32□C tumor - derived mutant forms of p53 (Ala143, His175, Trp248) fail to do so.

These results have been recently published:

Friedlander, P. Y. Haupt, Prives, C. and M. Oren. (1996) A mutant p53 that discriminates between p53 responsive genes cannot induce apoptosis. Mol Cell Biol. 16: 4961-4971.

Establishment of inducible mutant p53 in bone, lung and breast cell lines

A number of different experimental strategies have been employed for understanding the roles of wild-type and mutant forms of p53 in cell growth, arrest and death (reviewed in 1 and 2). Our approach has been to generate a number of cell lines that contain inducible wild-type or variant forms of p53 proteins. We utilized the tetracycline regulated system that was initially developed by Gossen and Bujard (15). This involves first establishing, through selection, a parental cell line that expresses a hybrid protein consisting of the tetracycline repressor fused to the VP16 activation domain. These are referred to as "15-1 neo" lines, referring to the plasmid and selection used for their construction. The chimeric protein that they express will bind to promoters containing tet repressor sites in the absence but not the presence of tetracycline. Derivatives of this cell line are then established containing constructs with the various p53 proteins desired expressed under promoters containing tet repressor binding sites. The cells are selected and maintained in the presence of tetracycline. We have established several inducible cell lines in p53 null Saos and H1299 cells, which are derived from osteosarcoma and lung tumors, respectively. We have also developed a 15-1 neo line in MDA-MB-H453 p53 null breast carcinoma cells and are in the process of establishing inducible p53 derivatives of that cell line. However, these cells are quite difficult to work with and so we have in parallel been characterizing the properties of the inducible Saos-2 and H1299 clones. Results from these clones have been informative both for wild-type and mutant forms of p53.

One cell line p53-7 when p53 is fully induced, undergoes massive apoptosis. However,interestingly, we have determined that within a given cell line the level of wild-type p53 can determine whether cells arrest or undergo apoptosis. We have analyzed the apoptotic response of several of these cell lines. Based on our experiments with both transient transfections and inducible cell lines it is clear that mutant forms of p53 are completely defective in inducing apoptosis. We have confirmed and extended the results of others (14) that the trans-activation function of p53 is not absolutely required for apoptosis. Interestingly, we also demonstrated that removal of the C-terminal 30 amino acids affects the apoptotic but not the arrest function of p53.

Part of these results have been recently published:

X. Chen, L. Ko L. Jayaraman, L. and C. Prives. 1996. p53 levels, functional domains and DNA damage determine the extent of the apoptotic response of tumor cells. Genes & Dev.10: 2438-2451.

Planned research:

Characterization of N-terminal antibody stabilization of mutant p53 DNA binding

Our future goals have evolved along with the results we have obtained over the 0past granting period. We planned to examine how modifiers of wild-type p53 function can affect mutant p53 DNA binding at 37□C. We found that while phosphorylation of wild-type p53 by cyclin dependent kinases stimulates and alters sequence specific DNA binding (16), similar phosphorylation of mutant p53 proteins neither stimulated DNA binding at 25□C or stabilized DNA binding at 37□C. Similarly while C-terminal fragments are capable of stimulating wild-type p53 DNA binding (17) their ability to increase DNA binding by mutant forms of p53 occurs at 25□C but not at 37□C. Thus, unlike the N-terminal specific antibodies, some C-terminal modifiers of wild-type p53 do not affect the temperature sensitive phenotype of mutant p53 proteins. We have therefore decided to focus more extensively on characterizing the effect of N-terminal specific antibodies. Two general approaches are planned: (1) We will mutagenize the PAb 1801 epitope region (residues 46-55) in the context of both wild-type and select tumor mutant forms of p53 and determine how residues within this region contribute to the temperature sensitive phenotype. (2) We will search for small peptides using phage display peptide libraries (18, 19) that mimic the effect of 1801 epitopes. By combining this search strategy with the 1801 epitope mutants constructed it is hoped to gain more understanding into the role of this region in temperature sensitive DNA binding. Importantly, if peptides are identified that have the same effect as the 1801-type antibodies, we may eventually be able to modify them for therapeutic purposes.

(2) **Characterization of mutant p53 function in inducible tumor cell lines**

There is considerable evidence that mutant forms of p53 can confer a gain of oncogenic function in tumor cells (eg. ref. 20). Our cell lines containing inducible forms of mutant p53 should be valuable in further understanding this process. We will ask the following questions:

1. Does the expression of tumor-derived mutant p53 proteins confer a growth and survival advantage upon the tumor cells ? To address this question cells expressing inducible p53 will be used to perform growth curves, FACS analysis of cell cycle distribution (as determined by DNA content), BRdU incorporation, and analysis of the expression of cell cycle regulatory proteins (through Western blotting).

2. Do mutant forms of p53 function as counter-apoptotic agents? The ability of mutant p53 proteins to inhibit apoptosis would provide a strong survival advantage to cells. We will determine if expression of the mutant p53 proteins blocks TNF-alpha, dexamethasone, and DNA damage (camptothecin) induced apoptosis.

3. Does the expression of the tumor derived mutant p53 proteins facilitate metastasis? This issue will be addressed initially through in vitro assays by analyzing the various stages of the metastatic pathway. Specifically, the Matrigel system (Becton Dickenson) will be used to determine the ability of cells to invade and pass through a basement membrane. The ability of the mutant proteins to induce angiogenesis will be ascertained through an "sandwich" assay composed of an endothelial cell layer, a free collagen layer, and a tumor cell(plus or minus mutant p53 expression) layer plated in 6-well dishes. If the mutant proteins induce angiogenesis then the endothelial cells would form cord like structures.

3. Identifying p53 interacting proteins which stabilize and restore wild-type function to tumor-derived mutant p53 proteins in *Saccharomyces cerevisiae.*

The budding yeast *Saccharomyces cerevisiae* does not contain a p53 homologue, despite the conservation of the cell cycle machinery from yeast to man (21). However, the ease of genetic analysis in yeast has proven fruitful in identifying p53 associated proteins, such as 53BP1 and 53BP2 (22), and in defining pathways through which p53 may act (23, 24). Human wild-type p53, when overexpressed from the galactose-inducible promoter in yeast, greatly reduce the growth rate of cells without affecting the response to heat shock or pheromone (25, 26). In contrast, most tumor-derived p53 mutants have either a slight or no effect on the growth rate of yeast (25,26). More importantly, constitutive expression of human wild-type p53 , but not tumor-derived mutant p53, is able to activate transcription of reporter genes under the control of a p53-binding site (27, 28).

Based on the observations of Ishioka et al. and with constructs kindly provided by Dr. Richard Iggo, we have developed a genetic screen that should allow the isolation of p53 interacting proteins. Budding yeast will be transformed with a reporter plasmid containing various p53-binding sites driving the expression of *HIS3* (e.g.. *RGC-DUAS1:GAL1:HIS3* on *TRP1/CEN*). Ishioka et al. (1993) demonstrated that this reporter is responsive to wild-type p53, by virtue of wild-type p53 binding to the RGC p53-binding sites, and allowing growth on histidine lacking medium (His^-). More importantly, Ishioka et al. (28) demonstrated that none of the tumor-derived p53 mutants tested were able to bind to and activate this reporter construct, hence, no growth on His^- medium. These reporter strains will be transformed with one of a number of hot-spot p53 mutant constructs (e.g.. *pADH:p53*^{R273H} on *LEU2/CEN*) or control vector. For the "transactivation assay", these strains are transformed with either a human or mouse cDNA expression library (*URA3* marked) which has been constructed from a cell line where the status of p53 is known. Preferentially, p53 should be either mutated or deleted to reduce the amount of false positives obtained when screening (wild-type p53 would bind to and activate the reporter). Additionally, we will use a high copy number yeast genomic library (*URA3* marked). Budding yeast may contain a protein(s) capable of binding to and restoring wild-type function to mutant p53. Regardless of the library used, transformants will be replica plated onto His^- medium and scored for those

colonies which grow at 30C, as well as 37C. The assumption is that only those library plasmids which encode for a protein(s) which can interact with mutant p53 and confer wild-type DNA-binding function will grow in the absence of histidine. However, the possibility exists that the library plasmid may encode a protein which binds to and activates the reporter construct on its own. To test this, all library plasmids isolated will be retransformed back into the reporter strain and assayed for their ability to grow on His^- medium. We are interested only in those library plasmids which require mutant p53 to grow on His^- medium. Additionally, the library plasmids isolated will be assayed for the ability to allow growth on His^- medium when other tumor derived mutants are present in the assay (e.g.: R175H, R248W, R249S). It is important to show that the library plasmid is not specific for one tumor derived mutant but for a family of tumor derived mutants.

Once a candidate clone(s) is obtained, it will be sequenced to determine if the gene(s) encodes a novel or previously identified protein(s). In particular, we are interested in a domain(s) common to the proteins that may suggest a common p53-binding motif. To confirm that the library plasmid(s) encode for proteins that associate with mutant p53, we will label cells with ^{35}S that express mutant p53 and contain one of the overexpressed library plasmids or a vector control. Immunoprecipitates of the extracts for mutant p53 with the monoclonal antibody PAb421 will be analyzed by SDS-PAGE and p53 bands detected by autoradiography. If the library plasmid(s) encode for a protein(s) that associates with mutant p53, we should detect a protein band present at higher levels in the mutant p53 immunoprecipitates as compared to the control vector. A new post-doctoral fellow in the laboratory, Dr. Charles DiComo has had extensive experience and success in developing and performing such genetic screens (29-31). Additionally, we have obtained or constructed the required strains, plasmids, and libraries required for these experiments. We will determine whether the clones obtained show specificity and restoration of wild-type p53 function *in vivo* in breast cell lines stably expressing tumor-derived p53 mutants at 37C. We will transfect p53 mutant-expressing stable cell lines MDA-MB-453 containing a biologically relevant p53 responsive reporter plasmid (e.g.: mdm2:CAT or p21:CAT) with our library plasmid(s) or a vector control. We will test whether the tumor derived p53 mutants, in the presence of the library plasmid(s), can activate transcription from these promoters at 37C. Our hope is that we can restore DNA binding function, similar to the restoration by PAb1801. The cell lines described above that express inducible tumor derived p53 mutants will be utilized as well. Our hope is that they restore wild-type function to an otherwise non-functioning p53 mutant at the physiological temperature. With these new proteins in hand, we can begin to understand the structural requirements necessary to convert a mutant p53 protein to a wild-type p53 protein. This will be achieved by a domain and deletion analysis of the new p53 associated proteins to determine the minimal region required to bind to the mutant p53 proteins.

CONCLUSION:

(1) We have identified a region of p53 that plays an important role in stabilizing the DNA binding conformation of p53 at physiological temperatures. Taken together our data show that mutant p53 proteins are conformationally flexible and suggest the possibility of developing small molecules that interact with the stabilization region we have identified in order to restore function to mutant p53 proteins under physiological conditions.

(2) It is proposed that there may exist distinct classes of p53 responsive promoters, whose ability to be activated by p53 can be regulated differentially. Such differential regulation may have functional consequences for the effects of p53 on cell fate.

(3) Inducible cell lines have been used to dissect the functions of p53 in cells. Our data show that the arrest and apoptosis functions of p53 are genetically separable. Our data suggest that transactivation is a component of a full apoptotic response, but that significant cell death (albeit with delayed kinetics) can occur when a transcriptionally defective p53 is expressed. Furthermore, deletion of the C-terminal 30 amino acids of p53 causes a marked decrease (but not complete loss) of the apoptotic function of p53. Since tumor-derived mutant forms of p53 contain intact N-and C-termini, our data that the complete failure of tumor derived mutants to induce apoptosis suggest that either DNA binding or an as yet unknown activity of the central portion of p53 is also essential for apoptosis in some cells.

progress during the past granting period:

Task 1: Examination of the properties of mutant p53 proteins:

(a) biochemical analyses of mutant p53 immunopurified proteins expressed from currently available baculoviruses: phosphate mapping, proteolysis protocols, hsp binding and oligomerization analyses

(a) We have examined the ability of mutant p53 proteins to be phosphorylated by cyclin dependent kinase, have examined thier state of oligomerization by sucrose gradient sedimentation analysis and non-denaturing gels. Neither of these tasks are ready for publication at present because the initial results did not provide any new insight into why mutants differ from wild-type protein with respect to DNA binding. With respect to examination of the effect of proteolysis, we did have some success, the results of which were pulbished in late 1993, i.e. very soon after the incipiation of the grant. Much of our work, as mentioned in the original report was predicated on our discovery of the temperature sensitive phenotype of mutant p53 proteins and so we have tried to further understand this property in biochemical terms. We have discoverd that wild-type p53 is also temperature sensitive such that it fails to resolve on non-denaturing gels at 37□C in contrast to experiments performed at 25□C. We observed that creatine phosphate will stabilize wild-type p53 at 37□C both for DNA binding and gel resolution. However, creatine phosphate does not stabiliz mutant p53 at 37□C.

(b) Cultured breast cell lines: develop purification procedures for mutant p53 from cells.

(c) comparative analyses of breast cell derived p53 proteins.

(b) We did not develop procedures for the isolation of mutant p53 from bresat cell lines because our preliminary experiments did not warrant this at the time. Thus, having not documented any significant differences with purified proteins it did not seem effective to proceed with this line of research.

(d) construction of baculoviruses expressing additional mutant p53s found in breast cells with aim of continuing comparative analysis of different mutant forms of p53..

(c) When we started our research on mutant p53 supported by DAMD17-94-J-4275 we had generated baculoviruses expressing four of the mutant forms of p53 as baculovirus expression vectors. Since then we have successfully constructed a fifth, ser 149, and are in the process of making the sixth (and final) "hotspot" mutant, ie mutated at codon 282. We have actually had a number of cloning difficulties with this, but are optimistic that we will solve our problem.

Task 2: Analysis of the DNA binding properties of mutant p53 proteins:

(a) Continue studies on the temperature sensitive phenotype of mutant p53 proteins performing gel-shift, DNase I footprinting and methylation interference assays. Examine exiting p53 response elements, adding more as they become available from the literature. As new mutants become available, determine the generality of the ts phenotype.

These studies have been completed and were published recently: Friedlander et al. J. Biol. Chem. 271: p.25468 (1996) We went on to examine the temperature sensitive phenotype *in vivo* as well and these results were included in the above paper. However, the results mandated a more careful analysis of the effects of transfected mutant p53 in tumor cells and these data yielded the interesting and important observation that one mutant, ala143, is defective in inducing apoptosis at 32□C in contrast to wild-type p53, but discriminates between p53 responsive genes. This was a collaborative study with M. Oren (Weizmann Institute) and was also recently published: Friedlander et al. Mol. Cell Biol. 16: p4961 (1996).

(c) Expand observations that PAb 1801 stabilizes DNA binding by mutant proteins: (i) generate mutants within the 1801 epitope region (ii) expression of N-terminal region of p53 with the aim of generating additional antibodies to further characterize the stabilization effect (iii) isolation of peptides from a phage display library that bind to the 1801 epitope region of p53 and testing whether they can stabilize mutant p53 DNA binding at 37□C.

We have recently initiated experiments designed to characterize the PAb 1801 epitope. We have made the very interesting observation that, in contrast to antibodies directed against other epitopes in p53, this antibody has a marked effect on the dissociation rate constant of p53 bound to its cognate DNA. We attempted repeatedly to generate a baculovirus expressing the N-terminus of p53 without success. This may be due to the observation that the N-terminus has very little structure and is very protease sensitive. However, we have made a construct expressing the N-terminus fused to GST and can cleave the N-terminus (amino acids 1-83) from the GST. We will attempt to generate antibodies to this N-terminal peptide.

(b) Search for consensus sites for mutant p53 proteins operating selection assays at both 25□C and 37□C. Once identified, use sites as response elements in reporter assays in insect cells (grown at 27□C) or mammalian cells at 37□C.

A graduate student, T. Zhang, spent one year trying to isolate by using an established PCR based selection protocol DNA sequences that are bound by mutant forms of p53. She was unable to get the protocol to work, and, based on the experience of other members of our laboratory, it may be that our PCR machine is not optimal for this type of procedure. We are hoping to purchase a newer and better PCR machine shortly (funds permitting) and are anxious to make a new stab at this. This becomes particulary important given our exciting observation that some mutant p53 proteins display altered promoter specificity.

Task 3. Identification of proteins or peptides that interact with mutant p53. Attempts to identify proteins that interact with mutant p53 are ongoing. In addition to using columns and immunoprecipitations from tumor cell lines, we have initiated a yeast screening program to identify factors that interact *functionally* with mutant p53 to restore its transcription function which is our penultimate goal. We already have some candidates that have passed our first set of criteria for *bona fide* mutant p53 modifiers or converters. I am extremely excited about these preliminary results. Even though these experiments do not not conform strictly to what we originally planned, they certainly conform in the best spirit of our intended goal which is to identify ways to convert mutant forms of p53 to the wild-type form in function. While we intend to pursue the other goals in parallel, ie identifying small peptides that can function in a similar matter, we need to find another collaborator or source of phage display libraries since our original collaborator, C. Siegal, is no longer working in this area. On a related note however, we had tried the effects of two peptides that had been idsolated thorugh screening a phage display library with our wild-type and mutant p53 proteins, and failed to see any effect of these peptides on either wild-type and mutant forms of p53 protein. We have been offered assistance by a colleague with experience in this area, and if necessary we will purchase a phage display library for these exepriments. It should be noted that we have made what I think is an important discovery regarding p53 in the past year, namely that there are a class of promoters that are activated by wild-type but not by mutant, apoptosis-defective forms of p53. Based on this observation, we must modify our original aim such that we ascertain which sites that mutants can bind to are relevant to induction of apoptosis. Thus, one good bet would be to require that a mutant be converted to a form that can bind to and activate the bax promoter. Although it is not clear whether bax induction is necessary and sufficient for apoptosis in breast cancer cells, this would still give an idea if we could convert mutant p53 to the most functional version of wild-type p53 as well.

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A Mutant p53 That Discriminates between p53-Responsive Genes Cannot Induce Apoptosis

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Human wild-type (wt) p53 can induce apoptosis in transiently transfected H1299 cells maintained at 37°C, whereas tumor-derived mutant forms of p53 (with the mutation Ala-143, His-175, or Trp-248) fail to do so. At 37°C, p53 with a mutation to Ala at amino acid 143 (p53Ala143) was transcriptionally inactive. However, at 32°C, p53Ala143 strongly activated transcription from several physiologically relevant p53-responsive promoters, to extents similar or greater than that of wt p53. Unexpectedly, p53Ala143 was defective in inducing apoptosis in H1299 cells at 32°C. Concomitantly with the loss of apoptotic activity, p53Ala143 was found to be deficient in its ability to activate transcription from the p53-responsive portions of the *Bax* and insulin-like growth factor-binding protein 3 gene promoters. It is proposed that there may exist distinct classes of p53-responsive promoters, whose ability to be activated by p53 can be regulated differentially. Such differential regulation may have functional consequences for the effects of p53 on cell fate.

The number of cells in a given tissue depends upon a delicate balance between cellular proliferation, growth arrest, and apoptosis. Deregulation of this balance by activation of oncogenes, inactivation of tumor suppressor proteins, or amplification of proteins which inhibit apoptosis can lead to the formation and progression of tumors. The p53 protein is an important player in this process (reviewed in reference 17), as about half of all human tumors produce aberrant p53 protein (41).

The levels and activity of p53 have been shown to increase in response to irradiation and other DNA-damaging agents (31, 46, 47, 57, 58). In the absence of functional wild-type (wt) p53, cells fail to arrest in G_1 in response to DNA damage (53). Activation of the p53 protein can affect cell fate through the induction of either growth arrest at G_1/S or G_2/M cell cycle checkpoints or apoptotic cell death (reviewed in references 28 and 30).

Cell cycle arrest and apoptosis by p53 appear to be differentially regulated functions. Murine hematopoietic cell lines undergo G_1 arrest in response to irradiation in the presence of interleukin 3, but when this survival factor is absent, p53-dependent apoptosis ensues (10, 26, 27). The uncoupling between growth arrest and apoptosis has also been demonstrated through the use of several mutant forms of p53 (42, 51, 65, 66). Furthermore, the apoptosis-blocking protein, bcl2, has been shown to prevent p53-mediated apoptosis without impairing p53-mediated G_1/S arrest (11, 14).

Studies with p53-null mice show that p53 is necessary for thymocytes to undergo apoptosis in response to DNA damage (12, 54, 56). Induction of apoptosis in fibroblasts by overexpression of c-*myc* requires wt p53 protein (38, 72). p53-dependent apoptosis can modulate the cytotoxicity of chemotheraputic agents (55) and can inhibit tumor growth and progression (70). Recently, hypoxia has been shown to induce p53-dependent apoptosis, and hypoxic conditions are believed to provide a selective pressure for p53 mutations (29).

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One well-studied characteristic of p53 is its ability to function as a DNA-binding dependent transcriptional activator (reviewed in reference 71). Transactivation by p53 is dependent on specific recognition of DNA sequences containing two copies of the motif 5′ Pu Pu Pu C(A/T)(T/A)G Py Py Py 3′. In addition, p53 is capable of repressing transcription in a manner that is independent of specific DNA recognition (24, 43, 59, 67, 69). Of central importance for growth arrest by p53 is transactivation of the *waf1*/p21 gene (7, 16). The product of this gene binds to and inactivates cyclin-dependent kinase complexes, exhibiting its most potent inhibitory effect on G_1 cyclin-dependent kinases (18, 20, 33, 78). A number of other p53-responsive genes, including *GADD45*, *mdm2*, the cyclin G gene, and the insulin-like growth factor-binding protein 3 gene (IGF-BP3), have been identified (reviewed in reference 50a).

With regard to apoptosis, the need for transactivation by p53 appears to depend on cell type. Certain cell types undergo p53-mediated apoptosis even in the presence of transcriptional inhibitors (9). Furthermore, a truncated p53 protein (residues 1 to 214), no longer capable of binding DNA, can induce apoptosis in HeLa cells (37) and Saos-2 cells (35) but not in H1299 cells (34). However, other studies have demonstrated that p53 transactivation function is indispensable for inducing apoptosis (66, 80).

One gene that is transactivated by p53 and which might be important for p53-mediated apoptosis is the *Bax* gene (60). The *Bax* promoter contains a p53-binding element which is sufficient for transactivation by wt p53 (60). Studies with transgenic *Bax*-null mice show that loss of *Bax* can cause hypoplasia in some tissues and hyperplasia in others (50). This variable phenotype may result from *Bax* being a member of a family of homologous proteins, the relative roles of each member of which may fluctuate by tissue and cell type. Importantly, however, thymocytes of *Bax*-null mice retain a p53-dependent response to DNA damage (50). Bax is a 21-kDa protein with 43% homology to bcl2 (61). Coimmunoprecipitation experiments show that *Bax* interacts with the bcl2 protein (61). bcl2 is an integral membrane protein localized to the inner mitochondrial, endoplasmic reticulum, and nuclear membranes (15, 39, 44, 52). Overexpression of bcl2 inhibits p53-mediated apoptosis (11, 21, 66, 74) and prevents the apoptosis seen with

deregulated c-*myc*, which is also believed to be p53 dependent (6, 22, 72). Overexpression of *Bax* accelerates the rate of cell death, probably through altering the ratio of *Bax* to bcl2 (60, 61).

In tumors, p53 mutation is frequently manifested as deletion of one p53 allele and a missense mutation in the other allele. The point mutations almost exclusively map within the DNA binding domain of p53, and the mutant p53 proteins are defective for transactivation and consequently growth suppression. One p53 mutant that has been detected in tumors contains a substitution of alanine for valine at amino acid 143. This mutant, p53Ala143, displays a temperature-sensitive phenotype, with functional sequence-specific DNA binding and transcriptional activation seen at the permissive temperature (32°C) but not at the restrictive temperature (37°C) (22a, 51, 84).

We report here that while wt p53 can induce apoptosis in transiently transfected H1299 cells at both 32 and 37°C, p53Ala143 fails to do so even at the permissive temperature. p53Ala143 does activate transcription from numerous physiologically relevant p53-responsive promoters at 32°C as efficiently as wild-type p53. Unexpectedly, however, p53Ala143 is very defective in its ability to activate transcription from a reporter construct containing the wt p53-responsive portion of the *Bax* promoter. This defect in *Bax* promoter transactivation may result, in part, from the very weak binding of p53Ala143 to the p53-binding element in this promoter. It is proposed that there may exist distinct classes of p53-responsive promoters. Qualitative and quantitative differences in the ability of p53 to regulate each class of promoters may have functional consequences for the decision of a cell to undergo apoptosis (or not) in response to p53 activation.

MATERIALS AND METHODS

Cells and transfections. H1299 cells were maintained at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Prior to transfection, cells were seeded at either 1.2×10^6 cells per 10-cm-diameter dish (for fluorescence-activated cell sorting [FACS] analysis) or 0.6×10^6 cells per 6-cm-diameter dish (for protein expression and luciferase assays). The medium was changed to Dulbecco modified Eagle medium supplemented with 10% FCS, and cells were transfected by the calcium phosphate method, with the precipitate left on cells for 6 h. Next, cells were glycerol shocked for 1 min and plated in RPMI 1640 supplemented with 10% FCS. After an additional hour at 37°C, cells were either left at 37°C or transferred to 32°C for the desired incubation time. Four and 2 μg of cytomegalovirus (CMV)-driven plasmid DNA were used for the transfection of 10- and 6-cm-diameter dishes, respectively. In luciferase assays, 1 μg of reporter plasmid was also included. When appropriate, DNA of the parental vector pCMVneoBam was used to keep the total amount of transfected DNA constant in each sample.

For luciferase assays, cells were rinsed with cold phosphate-buffered saline (PBS), resuspended in cell lysis buffer (Promega), and incubated for 15 min at room temperature. Samples were centrifuged, and the luciferase activity of the cleared supernatant was determined in the presence of luciferin (Promega) and ATP. Each transfection experiment was performed in triplicate.

For Western blot (immunoblot) analysis, H1299 cells were transfected with 1 μg of reporter construct plus 2 μg of pCMVp53wt, pCMVp53Ala143, pCMVp53His175, pCMVp53Trp248, pCMVp53Ser249, or pCMVp53His273. The total amount of DNA in each transfection mixture was kept constant by transfection of pCMVneoBam. Twenty-four hours after incubation at either 32 or 37°C, cells were collected and lysed. Total cellular protein was quantitated by the Bio-Rad assay, and 50 μg of protein of each sample was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting. Transfected p53 was probed with a mixture of PAb1801 and DO-1 and visualized with the aid of the Amersham ECL system.

Plasmids. Overexpression of wt or mutant p53 was achieved by transfection of pC53-SN3, pC53-249, pC53-SCX3, pC53-175, pC53-248, or pC53-273 (1, 48), all driven by the CMV early promoter/enhancer. The parental vector pCMVneoBam was used to keep the amount of transfected DNA constant among samples. To assay p53-dependent transactivation, the following reporter constructs were used: pGL2-NA(mdm2)-luc, containing the *Nsi*I-*Apa*I fragment of the murine *mdm2* gene (45) inserted into the pGL2-Basic vector (Promega); WWP-luc, containing 2.4 kb of the human *waf1*/p21 promoter and upstream region (20) (kindly provided by B. Vogelstein); pGL3-CyclinG-luc, containing 1.48 kb of the 5′ part of the rat cyclin G gene (82); pGL2-hmdm2-luc, comprising the *Pst*I-*Xho*I fragment (300 bp) of the human *mdm2* promoter cloned into pGL2-Basic (81); and pGL3-Bax-luc, containing a *Sma*I-*Sac*I fragment of the human *Bax* promoter (positions −687 to −318) (60) (kindly provided by J. Reed) cloned into the pGL3-Basic vector. pGADD45-luc contains the p53-binding element of the human *GADD45* promoter (described in reference 10a). IGF-BP3-BoxA-luc and IGF-BP3-BoxB-luc contain, respectively, box A and box B of the IGF-BP3 promoter cloned into pUHC13-3 (8) (kindly provided by L. Buckbinder and N. Kley).

Flow cytometry. For FACS analysis, adherent and detached cells were combined. Cells were fixed with methanol and resuspended in PBS at 4°C for 10 min. Cells were then incubated at room temperature with primary antibody DO-1. Cells were washed in PBS and treated with RNase A (50 μg/ml). The DNA was stained with propidium iodide (25 μg/ml; Sigma), and the cells were analyzed in a FACSORT cell sorter (Becton Dickinson). Cells were acquired and analyzed as described by Haupt et al. (36).

Purification of p53 proteins. Recombinant baculoviruses expressing wt and mutant p53 have been described elsewhere (4, 23). Extracts of infected Sf27 insect cells were prepared, and p53 was purified from lysates by immunoaffinity procedures (73). p53 protein was purified by using protein A-Sepharose columns cross-linked with the p53-specific monoclonal antibody PAb421 (32). All procedures were performed at 4°C to avoid thermal denaturation of p53 proteins. The proteins were eluted in buffer containing 50% ethylene glycol (73) and then dialyzed into buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 5 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol.

EMSA. Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (63). The following synthetic double-stranded oligonucleotides were used: *GADD45*, 5′ AATTCTCGAGCCCAGCATGCTTAGACATG GTTCTGCTCGAG 3′; cyclin G gene, 5′ AATTCTCGAGACGCAAGCCCGG GCTAGTCTCTCGGAG 3′; p21 gene, 5′ AATTCTCGAGGAACATGTCCCA ACATGTTGCTCGAG 3′; and *Bax*, 5′ GATCCTCACAAGTTAGAGACAAG CCTGGGCGTGGGCTATATTG 3′.

For EMSA experiments with fragments of DNA, a 132-bp *GADD45* fragment was generated by *Xba*I and *Kpn* digestion of pBluescript into which the *GADD45* oligonucleotide was cloned into the *Eco*RI site. A 93-bp probe containing the p53-binding element of the *Bax* promoter was generated by digestion of plasmid pGL3-Bax-luc with *Dde*I and purification of the appropriate fragment on a nondenaturing 6% polyacrylamide gel.

The probes were ^{32}P labeled by using the Klenow fragment of *Escherichia coli* DNA polymerase. The reaction mixtures contained 4 μl of 5× EMSA buffer (100 mM HEPES [pH 7.9]), 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM $MgCl_2$), 1 μl of 40 mM spermidine, 1 μl of 10 mM dithiothreitol, 1 μl of 0.5% Nonidet P-40, 1 μl of double-stranded poly(dI-dC) (60 μg/ml), 1 μl of bovine serum albumin (2 mg/ml), 3 ng of ^{32}P-labeled probe DNA, protein samples in dialysis buffer, and H_2O to 20 μl. The amounts of protein are noted in the figure legends, and reaction volumes were adjusted with dialysis buffer. Mixtures were incubated for 30 min at 25°C, then loaded on a nondenaturing 4% polyacrylamide gel containing 0.5× Tris-borate-EDTA buffer, 1 mM EDTA, and 0.05% Nonidet P-40, and electrophoresed in 0.5× Tris-borate-EDTA at 4°C at 180 to 200 V (not to exceed 40-mA current) for ~2 h.

DNase I protection assays. DNase I protection assays were performed as described previously (40). The 132-bp *GADD45* fragment described previously was ^{32}P labeled by using the Klenow fragment of *E. coli* DNA polymerase. The probes were incubated with purified baculovirus-expressed wt or mutant p53 at various concentrations for 30 min at room temperature and then subjected to DNase I digestion. The amount of DNase I used was pretested empirically to produce an even pattern of partial cleavage products. The DNase I reactions were performed on ice for 1 min and stopped by DNase I stop buffer (1% SDS, 20 mM EDTA, 200 mM KCl, 250 μg of yeast tRNA per ml). The samples were extracted with phenol-chloroform, and the DNA was precipitated with ethanol. The DNA fragments were then electrophoresed on a 10% polyacrylamide–7% urea gel.

RESULTS

Transient overexpression of p53Ala143 does not induce apoptosis in H1299 cells even at the permissive temperature. As previously reported (37), certain cell types do not require the sequence-specific transactivation (SST) activity of p53 in order to undergo p53-mediated apoptosis. However, p53 SST does appear to be required for apoptosis in the p53-null cell line H1299 (34). Since p53Ala143 has been shown to bind and transactivate p53 consensus sites at 32 but not 37°C, we elected to see if it could induce apoptosis at 32°C in H1299 cells. To study the effects of increased p53Ala143 levels in these cells, expression plasmids for human wt p53 and p53Ala143 were introduced by transient transfection using the calcium phos-

phate method. Hot spot mutant p53 proteins, p53His175 and p53Trp248, which are defective for transactivation at either temperature (see Fig. 3), were used as negative controls. All transfections were performed at 37°C in order to allow for efficient uptake of plasmid DNA by the H1299 cells. Cells were then either placed at 32°C or left at 37°C for the desired incubation period.

The effect of overexpressed p53 on the cell cycle distribution of transiently transfected H1299 cells was determined by flow cytometry (36, 80). First, p53 protein was detected and quantified with the p53-specific monoclonal antibody DO-1, followed by fluorescein isothiocyanate (FITC)-conjugated second antibody. Then the DNA content of individual cells was determined by propidium iodide staining, as a measure of cell cycle distribution.

Figures 1 and 2 show the effects of transient overexpression of wt p53 and p53Ala143 at 37 and 32°C, respectively. The upper left frames in Fig. 1A and B and 2A and B show the FITC intensities of individual cells as a function of cell size (forward scatter) in the total cell population of transiently transfected exponentially growing H1299 cells. By using a gate to define a region of high FITC intensity, cells expressing high levels of p53 were recorded separately (lower left frames). These cells represent the subpopulation of successfully transfected cells. The gating was set up so as to exclude unusually large cells believed to be cell doublets and multicellular clumps. In addition, very small cellular debris was also excluded through the use of an appropriate threshold parameter. Equal numbers of cells from the total culture and from the high-level p53-expressing subpopulation were acquired and analyzed. The cell cycle distributions of the total cell population (upper right frames of Fig. 1A and B and 2A and B) and of the high-level p53-expressing cells (bottom right frames) are shown.

At 37°C, wt p53 induced a significant increase in the sub-G_1 fraction (Fig. 1A and C), which represents apoptotic cells. At 38 h posttransfection, 15% of the high-level p53-expressing cells displayed sub-G_1 DNA content; at 50 h posttransfection, this number rose to 43% (Fig. 1C).

While wt p53 induced apoptosis at 37°C, expression of p53Ala143 did not increase the fraction of sub-G_1 cells more than expression of the hot spot mutants, p53His175 and p53Trp248 (Fig. 1C). Hence, p53Ala143 exhibited no significant apoptotic activity in H1299 cells maintained at 37°C.

That p53Ala143 does not induce apoptosis at the restrictive temperature (37°C) was expected, since under that condition the mutant protein is defective for DNA binding and transactivation (84) (data not shown). It was of interest, however, to determine the apoptotic effects of p53Ala143 at the permissive temperature of 32°C. It was found that p53Ala143 expression increased the sub-G_1 population only minimally. This effect was practically indistinguishable from that seen with the typical hot spot mutants p53His175 and p53Trp248 (Fig. 2B and C). By contrast, expression of wt p53 significantly increased the percentage of cells with sub-G_1 DNA content (Fig. 2A and C). The rate of increase was less than that seen at 37°C, but by 65 h at 32°C, the percentage of sub-G_1 cells approached that seen with cultures incubated for 50 h at 37°C (compare Fig. 1C and 2C). Hence, even at the permissive temperature of 32°C, p53Ala143 cannot induce apoptosis and behaves like other mutant p53 proteins.

p53Ala143 can transactivate numerous p53 response elements but is specifically defective in transactivating *Bax* and IGF-BP3 promoter elements. Since overexpression of p53Ala143 did not induce apoptosis at 32°C, we looked at the ability of this mutant to transactivate p53 response elements in H1299 cells. Zhang et al. (84) reported that p53Ala143 transactivates plasmids containing either an idealized p53 consensus binding site, p53CON, or the p53 consensus site located in the ribosomal gene cluster. Neither of these sites is derived from regulatory regions of identified p53 target genes. Thus, it remained possible that p53Ala143 is defective with respect to the activation of physiologically relevant p53-responsive genes. We therefore tested the abilities of different mutant forms of p53 to activate transcription, using a reporter construct containing the luciferase gene driven by the murine *mdm2* intronic promoter (3, 45). At 32°C, both wt p53 and p53Ala143, but not several hot spot mutant p53 proteins, transactivated this reporter (Fig. 3). In fact, the extent of transactivation by p53Ala143 was consistently greater than that of wt p53 (Fig. 3A and 4). In this experiment, the ratio of transfected plasmid DNA to cells was the same as that used in the apoptosis assays discussed above. In all transactivation experiments, cells were harvested 22 h after a temperature shift to 32°C in order to minimize the effect of cell loss through p53-mediated apoptosis. Western blot analysis of cells transfected with the p53-expressing plasmids revealed that the lack of transactivation by the p53 mutants was not due to decreased protein expression (data not shown; see Fig. 3B for a representative example). Indeed, the mutants were expressed at higher levels than was wt p53.

To assess the generality of these observations, we compared the relative abilities of wt p53 and p53Ala143 to activate several additional authentic p53-responsive promoters in transfected H1299 cells. At 32°C, p53Ala143 activated, to the same extent as or greater extent than wt p53, the transcriptional regulatory regions of the human *mdm2* gene, the human *waf1*/p21 gene, and the rat cyclin G gene (Fig. 4). In these experiments, the ratio of transfected plasmid DNA to cells was the same as that used in the apoptosis assays discussed above. Experiments in which a range of p53 expression plasmid DNA amounts were transfected revealed that the values shown here are plateau levels of transactivation (data not shown). Additionally, p53Ala143 and wt p53 activated to similar extents transcription of a reporter construct containing the luciferase gene driven by a minimal promoter linked to the *GADD45* p53-binding element (Fig. 4).

Two striking exceptions were identified when we used constructs containing the proximal region of the p53-responsive *Bax* promoter, including the p53 binding sequence (60) or p53 response element (box A and box B) in the IGF-BP3 promoter (8). In this case, p53Ala143 consistently transactivated these reporters to a much lesser degree than did wt p53 (Fig. 4). Furthermore, this quantitative difference was also observed clearly when decreasing amounts of p53 expression plasmids were transfected into H1299 cells (data not shown).

One possible explanation for the discrepancy between wt p53 and p53Ala143 was that activation of *Bax* or IGF-BP3 requires more p53 protein, and p53Ala143 accumulates to lower levels than wt p53 and is thus present in insufficient amounts. However, Western blot analysis of cells transfected with p53 expression plasmids and a reporter construct and incubated at either 32 or 37°C revealed that the p53Ala143 protein was expressed at 32°C to the same level as wt p53 (Fig. 3B). Consequently, the relatively weak activation by p53Ala143 of the constructs containing the *Bax* and IGF-BP3 promoter elements was not due to limited levels of protein expression.

The p53Ser249 mutant exhibited very high levels of expression at both 37 and 32°C, while the level of wt p53 expression was also constant but significantly lower (Fig. 3B). However, significantly more p53Ala143 was expressed at 37°C than at 32°C, with the level approaching that of p53Ser249 at 37°C and

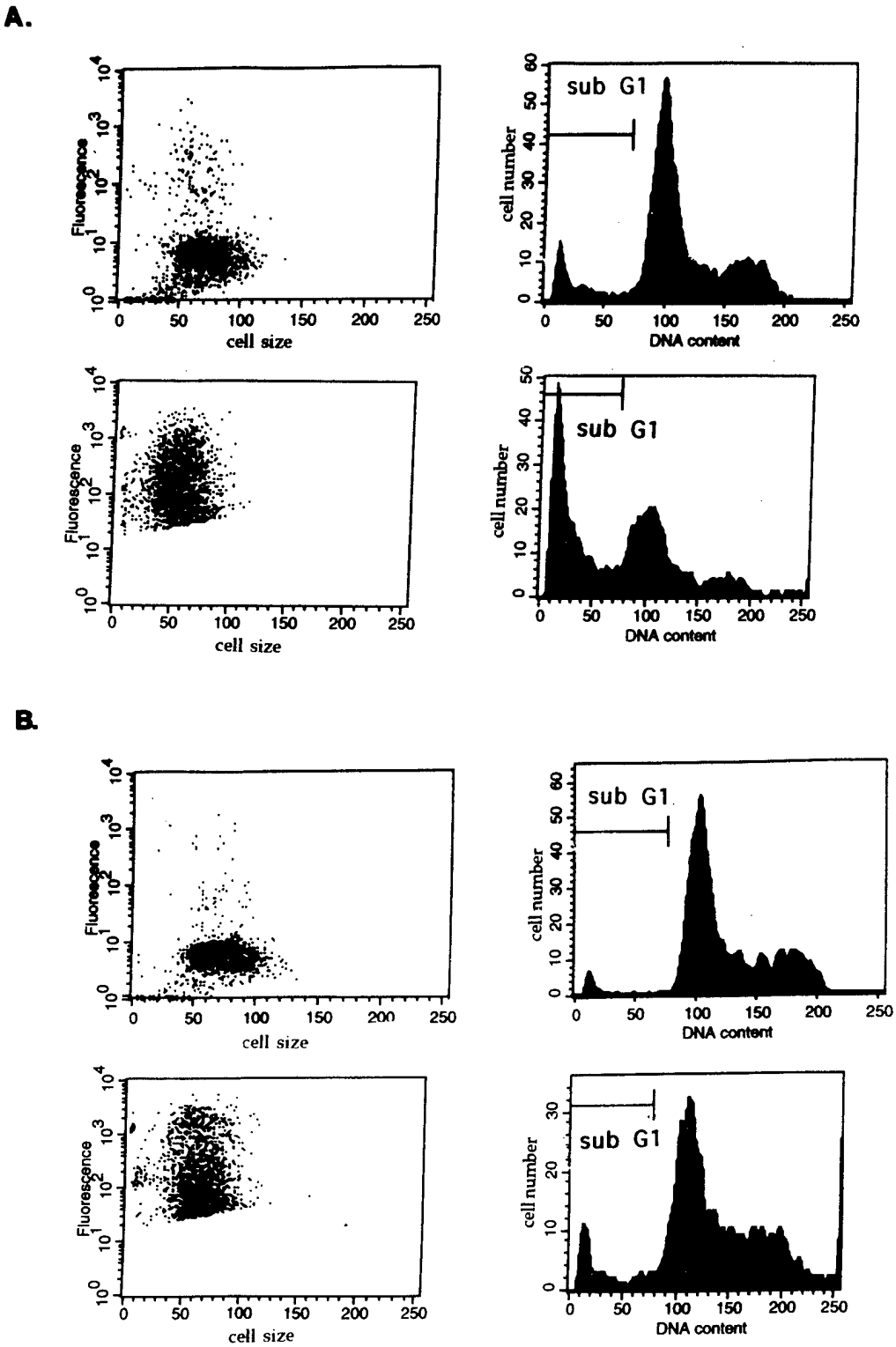

FIG. 1. Overexpression of wt but not mutant p53 proteins induces cell death in transiently transfected H1299 cells at 37°C. Shown is flow cytometric analysis of H1299 cells transiently transfected with a CMV-driven plasmid expressing wt p53, p53Ala143, p53Trp248, or p53His175. All cells were transfected and incubated for either 38 h (C) or 50 h (A to C) at 37°C. Cells were stained for p53 and analyzed for FITC intensity (fluorescence) as a function of cell size, and patterns are shown for the total unsorted cell population (top left frames in panels A and B) and the successfully transfected, high-level p53-expressing cells (bottom left frames). A total of 2,000 events from each population were acquired. Note that p53 fluorescence is plotted on a logarithmic scale. DNA content as determined by propidium iodide staining was used to ascertain the cell cycle distribution for the total cell population (upper right frames in panels A and B) and the transfected high-level p53-expressing cells (lower right frames). Results are shown for samples incubated for 50 h at 37°C following transfection with a CMV-driven wt p53 expression plasmid (A) or p53Ala143 expression plasmid (B). (C) The percentage of cells with a sub-G_1 DNA content is shown graphically for cells incubated for either 38 or 50 h at 37°C following transfection with a CMV-driven plasmid expressing wt p53, p53Ala143, p53His175, or p53Trp248.

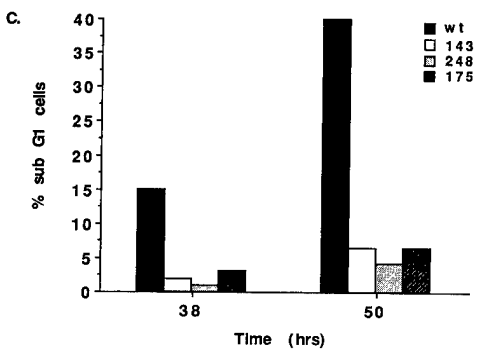

FIG. 1—*Continued.*

that of wt p53 at 32°C (Fig. 3B). This result is consistent with mutant p53 proteins being more stable than wt p53 and with p53Ala143, but not p53Ser249, exhibiting a temperature-sensitive phenotype (25).

These results demonstrate that p53Ala143 is capable of transactivating numerous physiologically relevant p53-responsive promoters as efficiently as wt p53. However, p53Ala143 is specifically defective in activating other p53-responsive promoters derived from the *Bax* and IGF-BP3 genes. Hence, this mutant form of p53 can discriminate between different p53 target genes.

p53Ala143 binds very weakly to the *Bax*-binding element. Since p53Ala143 activates transcription from the *Bax* promoter to a significantly lesser degree than wt p53, we examined the ability of this p53 mutant to bind to the *Bax* p53 response element. The p53 proteins used in these assays were immunoaffinity purified from Sf27 insect cells infected with appropriate recombinant baculoviruses. (The insect cells were maintained and infected at 26°C, and extraction and purification were conducted at 4°C. Thus, both the mutant and wt proteins are not likely to be thermally inactivated at any stage of their preparation.) EMSAs were performed to determine the relative abilities of wt p53 and p53Ala143 to bind to short oligonucleotides containing the p53 binding sites found in the *waf1*/p21, *GADD45*, cyclin G gene, and *Bax* promoters. We observed that p53Ala143 bound more weakly to all of the oligonucleotides tested, displaying 25 to 45% of the extent of binding seen with wt p53 (Fig. 5A and B). Similar results were obtained when an oligonucleotide containing the ribosomal gene cluster (RGC) binding site was used (data not shown). This finding suggests that under the conditions of the EMSA, p53Ala143 was still partially defective for DNA binding. Strikingly, however, the wt p53 protein itself displayed a significantly lower affinity for the *Bax* site compared with the other oligonucleotides tested (Fig. 5A and data not shown). As seen with the other sites, the binding of p53Ala143 to the *Bax* site oligonucleotide was also less than that of wt p53, and thus its overall binding to this site was extremely weak. Note, however, that these data do not show that p53Ala143 is specifically defective for binding to the *Bax* element. Competition experiments with unlabeled wt and mutant *GADD45* oligonucleotides demonstrated that the binding to the *Bax* fragment by p53Ala143, although very weak, was nevertheless sequence specific (data not shown).

We next examined p53 binding to larger fragments encompassing the p53 binding sites. DNase I footprinting analysis revealed that wt p53 and p53Ala143 made identical contacts with the 132-bp fragment containing the *GADD45* p53 binding site, since they displayed similar patterns of protection and hypercutting (Fig. 5C). Consistent with the less efficient binding by p53Ala143 seen by EMSA, approximately threefold more p53Ala143 than wt p53 was required for protection of the *GADD45* fragment from DNase I. Also in agreement with the data obtained with short synthetic oligonucleotides, the larger *GADD45* fragment was bound more effectively by wt p53 than was a 93-bp fragment containing the *Bax* promoter region including the p53 binding site. The slower mobility of the p53-*Bax* fragment complexes may be related to the possibility of unusual structural features caused by an unusually long stretch of A's in the *Bax* promoter fragment (60) (see Discussion). Significantly, the binding of p53Ala143 to the *Bax* fragment was extremely poor (Fig. 5D and E).

DISCUSSION

In the study described here, transient-transfection assays were used to evaluate the relationship between p53-induced apoptosis and the ability of p53 to activate the transcription of various target promoters. Transient transfection of p53 expression plasmids can induce apoptosis in a variety of cell types (34, 36, 37, 80). This assay typically entails the delivery of the transfected plasmid DNA into the cells by the calcium phosphate method. This procedure probably imparts a strong DNA damage signal (64, 79) which may be required for allowing the transfected p53 to serve as an efficient effector of apoptosis. In support of this view, a particular p53 mutant (p53Gln22,Ser23) causes apoptosis in transiently transfected cells (37) but not in cells which express it from a stably integrated transgene (66).

As reported previously, the SST function of p53 is dispensable for the apoptotic activity of excess p53 in certain cell types (9, 72), including HeLa (37) and Saos-2 (35) cells. However, in other cell types, retention of SST appears to be tightly correlated with p53-mediated apoptosis and may therefore be obligatory for this response. This latter category includes the human lung carcinoma-derived cell line H1299 (34). It could therefore be expected that SST-competent forms of p53 will be capable of eliciting efficient apoptosis in H1299 cells.

Surprisingly, p53Ala143, which can activate transcription in a temperature-sensitive manner in transiently transfected H1299 cells, was unable to induce apoptosis in the same cells at the permissive temperature of 32°C. A similar observation was recently described with respect to K562 cells; in that case, p53Ala143 failed to induce apoptosis despite activating the *waf1* gene (51). However, in our study, more extensive examination of the SST ability of p53Ala143 revealed that while it activates strongly several p53-responsive promoters at 32°C, this mutant is defective in transactivating the *Bax* and IGF-BP3 (box A and box B) promoter elements. This observation might also explain several earlier reports documenting the surprising failure of SST-competent p53 mutants to exhibit transformation-inhibitory activity (13, 42, 62). It now appears that at least in some common assays of p53's tumor suppressor function, it is the apoptotic rather than growth-arresting activity of p53 which is crucial (37, 65).

The deficient transactivation of the *Bax* promoter by p53Ala143 may be due, at least in part, to the very low affinity of this mutant p53 for the *Bax* p53-binding element. Under the conditions of the EMSA, p53Ala143 exhibited a reduced interaction with all p53 target sites tested. This finding might imply that under these in vitro conditions, the conformation of p53Ala143 was still not fully identical with that of authentic wt p53. However, whereas other p53 binding sites were still bound by p53Ala143 with a fairly high affinity, the binding of this mutant to the *Bax* site was remarkably poor. This very weak

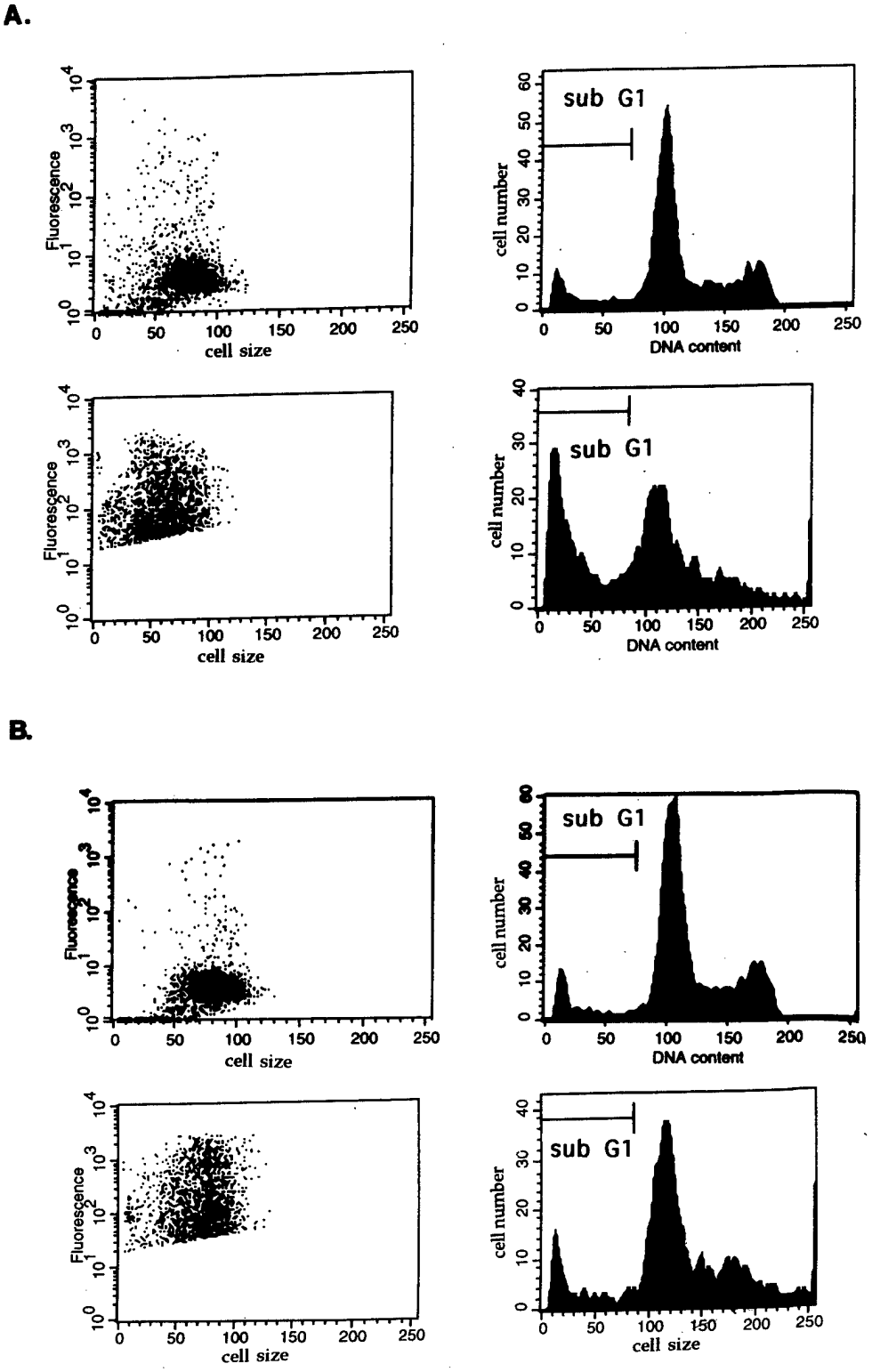

FIG. 2. The mutant p53 protein p53Ala143 does not induce apoptosis in transiently transfected H1299 cells at 32°C. Shown is flow cytometric analysis of H1299 cells transiently transfected with a CMV-driven plasmid expressing wt p53, p53Ala143, p53Trp248, or p53His175. All cells were transfected at 37°C and incubated for either 25 h (C), 50 h (C), or 65 h (A to C) at 32°C. Cells were stained and analyzed as for Fig. 1. Results are shown for samples incubated for 65 h at 32°C following transfection with a CMV-driven wt p53 expression plasmid (A) or p53Ala143 expression plasmid (B). (C) The percentage of cells with a sub-G_1 DNA content is shown graphically for cells incubated for 25, 50, or 65 h at 32°C following transfection with a CMV-driven wt p53, p53Ala143, p53His175, or p53Trp248 expression plasmid.

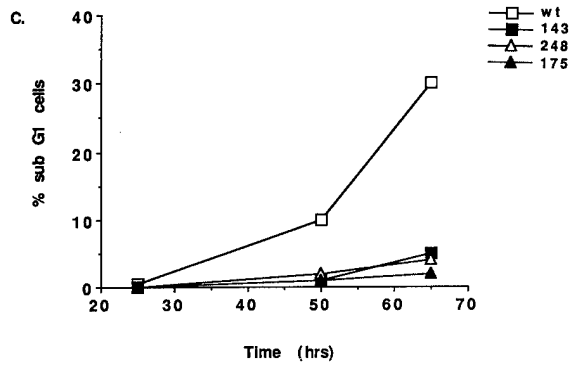

FIG. 2—*Continued.*

binding correlates well with the very minimal, albeit detectable, activation of the *Bax* reporter by p53Ala143.

It is of interest that wt p53 itself revealed lower affinity for the *Bax* site than for sites from other p53-responsive promoters. Nevertheless, wt p53 activated the *Bax* promoter very efficiently. One interpretation of these data is that there is a threshold for DNA binding by p53, which is sufficient for full transcriptional activation in vivo. The binding of p53Ala143 to the *Bax* fragment may be well below this threshold and therefore insufficient for effective transactivation.

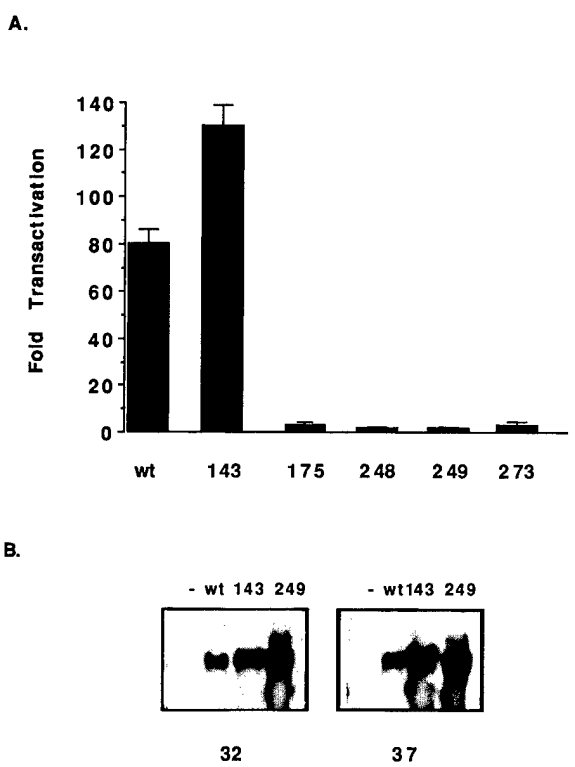

FIG. 3. Relative transactivation at 32°C of the *mdm2* promoter by wt and mutant p53 proteins in transfected H1299 cells. (A) H1299 cells were transiently transfected with the indicated p53 expression plasmid and the murine *mdm2*-luciferase reporter construct. Cell extracts were prepared following 22 h of incubation at 32°C as described in Materials and Methods. Luciferase activity is represented as fold transactivation relative to samples transfected with reporter construct plus non-p53-expressing control plasmid. The fold activation represents an average of triplicate samples. The standard deviation is also indicated. The p53 proteins expressed from the transfected CMV-driven plasmids (from left to right, wt p53, p53Ala143, p53His175, p53Trp248, p53Ser249, and p53His273) are indicated on the horizontal axis. (B) Representative example of levels of p53 protein expression following 22 h of incubation of transiently transfected H1299 cells at 32 or 37°C. Fifty micrograms of total cellular protein as determined by the Bio-Rad assay was resolved by SDS-PAGE and analyzed by Western blotting with a mixture of PAb1801 and DO-1 used as probes. In this example, cells were transfected with a *Bax* reporter construct plus wt p53, p53Ala143, p53Ser249, or pCMVneoBam as vector control.

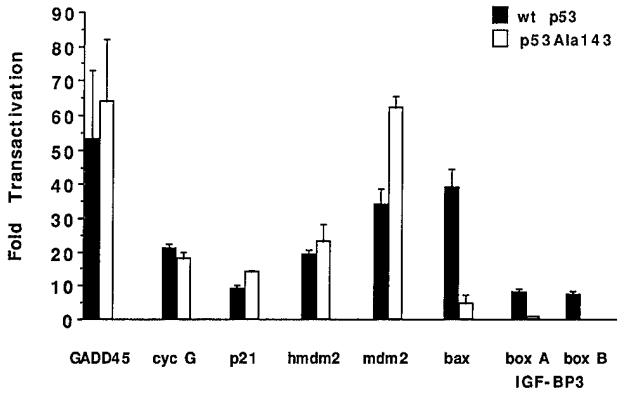

FIG. 4. Relative transactivation of various p53-responsive promoters by wt p53 and p53Ala143 at 32°C in transiently transfected H1299 cells. H1299 cells were transiently transfected with the indicated expression plasmid and p53-responsive reporter construct. Cell extracts were prepared following a 22-h incubation at 32°C and analyzed as described in Materials and Methods. Fold transactivation represents the luciferase activity relative to values obtained with transfection of the same reporter construct with a control non-p53-expressing plasmid. All values represent the average of triplicate samples; the standard deviation is indicated. The reporter constructs examined were GADD45-luc (GADD45), pGL3-CyclinG-luc (cyc G), pGL3-p21-luc (p21), pGL2-hmdm2-luc (hmdm2), pGL2-mdm2-luc (mdm2), pGL3-Bax-luc (bax), IGF-BP3-BoxA-luc (box A), and IGF-BP3-BoxB-luc (box B). The p53 expression plasmids studied were CMV-driven wt human p53 and CMV-driven p53Ala143.

The *Bax* promoter does not contain a typical consensus p53-binding element, i.e., two adjacent 10-bp consensus sites (19, 60). Rather, this region contains one perfect 10-bp consensus site flanked on the 5′ side by a sequence with 7 of 10 matches and on the 3′ side by two imperfect 10-bp motifs (7 and 8 bp out of 10 bp). This lack of an ideal consensus site may account for the weaker binding by wt p53.

On the other hand, the lack of correlation between relative binding and activation of the *Bax* promoter by wt p53 may reflect complex regulation of the *Bax* gene. It is conceivable that full activation of the *Bax* gene by p53 requires interactions with one or more proteins which are not relevant for other p53 target genes. The mutation at codon 143 might thus alter the p53 protein in a global way, affecting not only its DNA binding specificity but also its cooperative interactions with these other proteins. This possibility can be considered as an alternate or contributing factor for explaining the specific defect of p53Ala143 in *Bax* transactivation.

Interestingly, upstream of the p53 binding element in the *Bax* gene is a long string of A residues. This segment may induce structural alterations such as DNA bending within this region. Furthermore, the high AT content might permit dissociation of the DNA strands. The resultant single strands may then recruit p53 protein to the promoter region through the affinity of p53 for single-stranded DNA (2, 5, 77). DNA damage increases the level of p53 protein in a cell, possibly enhancing the recruitment of p53. This may allow for greater than expected specific binding of p53 to the *Bax* element in cells with damaged DNA. In line with this model, comparable amounts of transcriptionally competent p53 were found to be potent inducers of endogenous *Bax* gene expression only in irradiated cells, whereas *waf1* gene expression was elevated

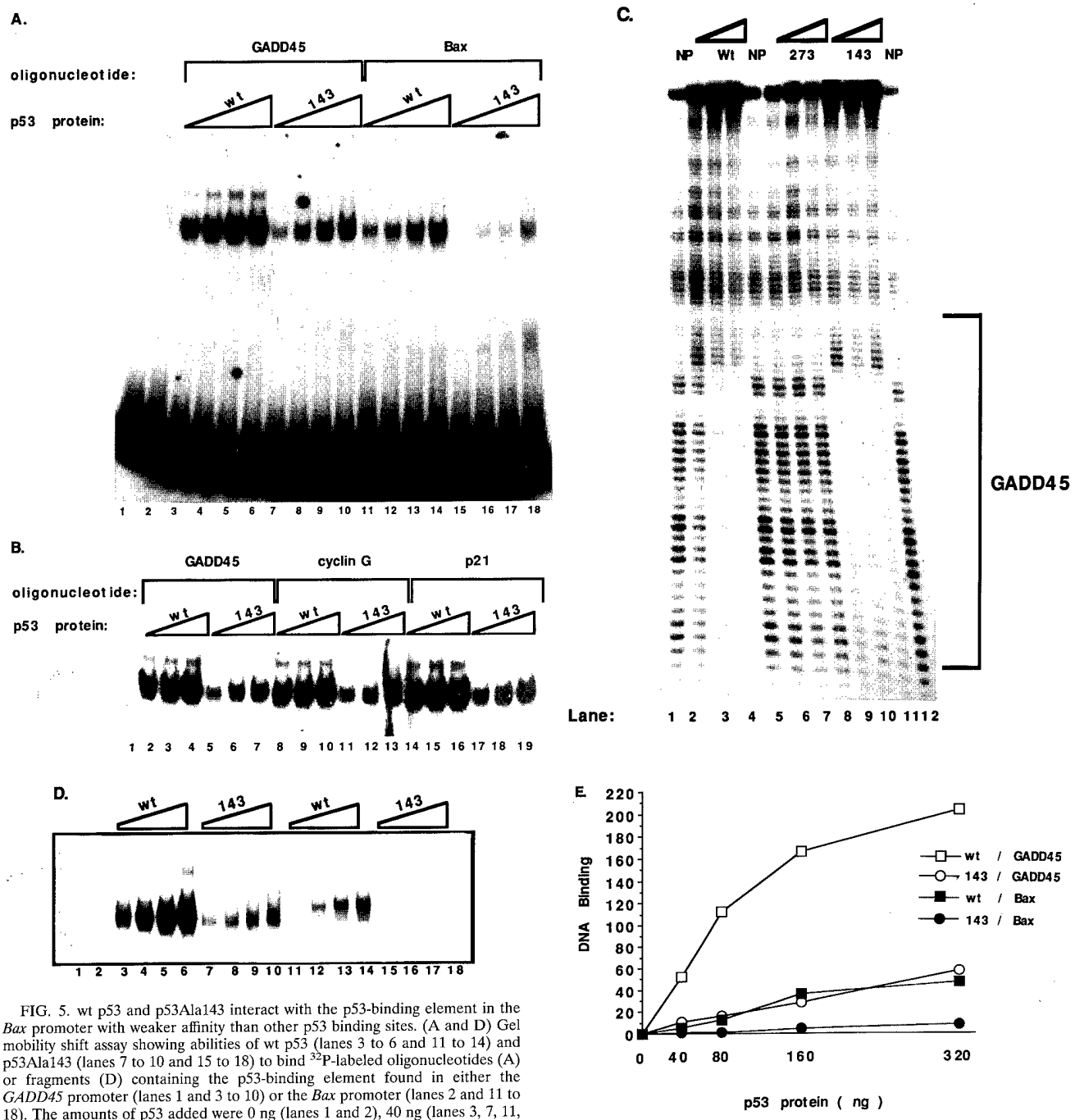

FIG. 5. wt p53 and p53Ala143 interact with the p53-binding element in the *Bax* promoter with weaker affinity than other p53 binding sites. (A and D) Gel mobility shift assay showing abilities of wt p53 (lanes 3 to 6 and 11 to 14) and p53Ala143 (lanes 7 to 10 and 15 to 18) to bind ^{32}P-labeled oligonucleotides (A) or fragments (D) containing the p53-binding element found in either the *GADD45* promoter (lanes 1 and 3 to 10) or the *Bax* promoter (lanes 2 and 11 to 18). The amounts of p53 added were 0 ng (lanes 1 and 2), 40 ng (lanes 3, 7, 11, and 15), 80 ng (lanes 4, 8, 12, and 16), 160 ng (lanes 5, 9, 13, and 17), and 320 ng (lanes 6, 10, 14, and 18). (B) Gel mobility shift assay showing abilities of wt p53 (lanes 2 to 4, 8 to 10, and 14 to 16) and p53Ala143 (lanes 5 to 7, 11 to 13, and 17 to 19) to bind ^{32}P-labeled oligonucleotides found in the *GADD45* promoter (lanes 2 to 7), the cyclin G gene promoter (lanes 8 to 13), or the p21 promoter (14 to 19). Lane 1 represents a reaction with no p53 protein and using the *GADD45* oligonucleotide. (C) DNase I footprinting analysis of wt p53, p53Ala143, and p53His273 proteins. A ^{32}P-labeled fragment containing the *GADD45* p53 binding site was incubated with 250 ng (lane 2), 500 ng (lane 3), or 800 ng (lane 4) of wt p53 protein and 1,500 ng (lanes 6 and 9), 2,000 ng (lanes 7 and 10), or 2,500 ng (lanes 8 and 11) of either p53His273 or p53Ala143 protein. NP, reactions to which no p53 protein was added (lanes 1, 5, and 12). (E) Graphical representation of DNA binding by wt p53 and p53Ala143 to the *GADD45* and *Bax* fragments. DNA binding units represent the values (10^3) obtained by quantification of shifted bands by using ImageQuant phosphor imaging. Equivalent specific activities of the ^{32}P-labeled *GADD45* and *Bax* fragments, as determined by acid precipitation, were used.

strongly by the same amounts of p53 even without irradiation (27). Furthermore, maximal induction of endogenous *Bax* RNA, but not *GADD45* RNA, was seen in irradiated cells under conditions in which they presumably expressed only submaximal levels of p53 (83) despite the apparently lower affinity of wt p53 to the *Bax* site (this report). Thus, activation of the *Bax* promoter by p53 may be favored in cells exposed to DNA damage, overriding the inherent low affinity of p53 for this promoter. While the mechanism of this preferential activation remains unclear, it is conceivable that p53Ala143 is defective also in some aspect of this mechanism.

The correlation between transactivation of the *Bax* promoter and induction of apoptosis suggests that the *Bax*-encoded protein may play a role in p53-mediated apoptosis. This suggestion is consistent with earlier reports (59, 60, 68, 83) and with the documented apoptotic effect of *Bax* overexpression (61). However, the exact relationship between physiological p53-dependent apoptosis and *Bax* overexpression remains unresolved. In particular, it is of note that the apoptotic death of irradiated thymocytes, which is strongly p53 dependent (12, 56), takes place with apparently normal kinetics in Bax-null (knockout) mice (50). It is therefore possible that the relative role of *Bax*, as well as of the other members of the Bcl2 family, will vary with cell type and specific environmental cues (reviewed in reference 76). This possibility also agrees with the variable phenotype of both hyperplasia and hypoplasia in *Bax*-null mice (50).

Our data for the IGF-BP3 promoter elements demonstrate that the transactivation defect of p53Ala143 is clearly not limited to *Bax*. While *Bax* overexpression is linked to the apoptotic response, the role of IGF-BP3 in cell death is less well established. However, IGF-BP3 antagonizes the activity of insulin-like growth factor 1 (IGF-1), and lowered activity of IGF-1 or reduced levels of IGF-1 receptor have been shown to result in apoptosis in some cases (reviewed in reference 4a). Indeed *Bax* and IGF-BP3 may be members of a subset of p53 target genes, all of which are impaired with regard to transactivation by this mutant at 32°C. Failure to activate any of these putative genes might underlie the inability of p53Ala143 to elicit apoptosis in H1299 cells. If this is the case, it is conceivable that reconstitution of normal *Bax* expression may not, by itself, suffice to restore full apoptotic competence to p53Ala143. It will be of great interest to identify additional p53-responsive genes which exhibit a selective deficiency in transactivation by p53Ala143; such genes may play an important role in mediating p53-dependent apoptosis, at least in cells in which the SST function is clearly required for this biological activity.

The properties of p53Ala143 are very reminiscent of those ascribed to another p53 mutant, p53Pro175. Like p53Ala143, p53Pro175 is a potent transactivator of many target promoters, yet it does not inhibit oncogene-mediated transformation (13). A recent study has revealed that p53Pro175, too, is incapable of triggering apoptosis in transiently transfected H1299 cells (65). This contrasts with its ability to act as an effective blocker of cell cycle progression, which is consistent with the efficient activation of the *waf1* promoter and possibly also the promoters of other growth-related genes (65). Importantly, p53Pro175 was also recently found to be severely impaired with regard to activation of the *Bax* and IGF-BP3 promoters in transiently transfected cells (57a). This observation defines a class of transcriptionally competent p53 mutants which share the inability to trigger apoptosis and to induce transcription from the *Bax* promoter and strengthens the conviction that these two properties are related.

Finally, our data suggest the existence of discrete classes of p53-responsive genes. Although the experiments reported here and in parallel by Ludwig et al. (57a) were carried out with mutant forms of p53, it is tempting to speculate that a similar promoter discriminatory capacity is inherent to wt p53. This model would propose that wt p53 possesses a potential for the differential recognition of distinct subclasses of target genes. Dependent upon the specific environmental cues and intracellular context, p53 might interact with and activate either its entire panel of target genes or only a subset thereof. Interestingly, a temperature-sensitive p53 protein, p53Val135, induced equal amounts of *waf1*/p21 mRNA but very different amounts of *mdm2* mRNA when introduced into different cell lines (49). Indeed, it has been demonstrated that phosphorylation of p53 by S-phase and G_2/M-phase cyclin-dependent kinases stimulates binding to only a subset of p53 response elements (75). This observation suggests the possibility that p53 structure can be regulated such that binding site selectivity is altered. Possibly mutant forms of p53 are incapable of such regulation. This differential recognition and activation may explain how stimulation of wt p53 can lead to apoptosis in certain situations and to viable growth arrest in others.

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Regulation of Mutant p53 Temperature-sensitive DNA Binding*

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We have examined in detail the DNA binding properties of several immunopurified tumor-derived mutant p53 proteins (Val-143 → Ala, Arg-175 → His, Arg-248 → Trp, Arg-249 → Ser, and Arg-273 → His). While all mutants were defective for binding to DNA at 37 °C, each bound specifically to several cognate p53 binding sites at sub-physiological temperatures (25–33 °C), and several mutants activated transcription from a p53-responsive promoter at 26 °C in transfected H1299 cells. Heating mutant p53 proteins at 37 °C irreversibly destroyed their ability to subsequently bind at 25 °C. However, several different monoclonal antibodies that each share the ability to recognize an epitope encompassing amino acids 46–55 markedly stabilized binding by mutant p53 proteins at 37 °C. Both intact antibody and FAb fragments allowed mutant p53 to bind to DNA. By contrast, antibodies that recognize epitopes located elsewhere within p53 stabilized mutant p53 binding significantly less effectively. Our data show that the major hot-spot p53 mutants have the intrinsic ability to bind to DNA and that a unique region within the N terminus of p53 may be critical for rescuing them from loss of binding at physiological temperatures. This suggests the possibility of developing small molecules that can stabilize mutant p53 proteins under physiological conditions.

Induction of the p53 pathway in cells, initiated by damage to DNA, leads to cell cycle arrest or apoptosis (Donehower and Bradley, 1993; Levine, 1993; Haffner and Oren, 1995; Ko and Prives, 1996). Disruption of this pathway is highly correlated with the development of the tumorigenic phenotype. One function of p53 that is likely to be involved in its role as a tumor suppressor is its ability to activate transcription of genes containing p53 response elements. Such elements contain a repeat of the sequence 5′-RRRC(A/T)(T/A)GYYY-3′ (reviewed by Vogelstein and Kinzler, 1992). There are a number of genes that contain these binding sites that are activated when cells are induced to express high levels of wild-type p53. Among these are GADD45 (Kastan *et al.*, 1992), mdm2 (Wu *et al.*, 1993), WAF 1/p21/CIP 1 (El-Dierry *et al.*, 1993), cyclin G (Okamoto and Beach, 1994), bax (Miyashita and Reed, 1995), and IGFBP3 (Buckbinder *et al.*, 1995). Expression of each of these genes is likely to be relevant in some way to the p53 pathway in cells.

Three functional domains of p53 have been identified. At the N terminus is a transcriptional activation region (amino acids 1–43); within the central part is the sequence-specific DNA binding domain (amino acids 100–300), and the C-terminal portion contains an oligomerization region (amino acids 319–360) that forms tetramers (reviewed by Prives, 1994). Additionally, it is clear that sequences and signals outside of the DNA binding domain, especially within the C terminus, regulate the function of the DNA binding domain of wild-type p53 (Hupp *et al.*, 1992; Halazonetis and Kandil, 1993; Wang and Prives, 1995).

Mutation of the p53 gene occurs in approximately 50% of the tumors derived from the major forms of human cancer (reviewed in Hollstein *et al.* (1991, 1994) and Nigro *et al.* (1989)). p53 genomic mutation is usually manifested as deletion of one allele coupled to a missense mutation in the other allele. The result of two such mutagenic events is a tumor cell that expresses exclusively mutant p53 protein, frequently at very high levels. Strikingly, nearly all of the >2000 tumor-derived mutations identified to date are located within the central DNA binding domain. While the majority of residues within this region have been mutated, there are six hot-spots that lie within conserved region III (Arg-175), IV (Gly-245, Arg-248, and Arg-249), and V (Arg-273 and Arg-282). Mutation at these codons occurs with unusually high occurrence, *i.e.* at frequencies ranging between 4 and 10% of the total number of mutations. Thus, together around 40% of the p53 mutations that have been identified involve a hot-spot site.

The fact that the tumor-derived mutations are distributed almost exclusively within the DNA binding domain underscores the importance of DNA binding to the normal function of p53. Indeed, a number of studies have characterized the function of tumor-derived p53 mutants with respect to their sequence-specific DNA binding and transactivation activities (Kern *et al.*, 1991a; Bargonetti *et al.*, 1991, 1992; Unger *et al.*, 1992; Chen *et al.*, 1993; Chumakov *et al.*, 1993; Miller *et al.*, 1993; Zhang *et al.*, 1993; Pietenpol *et al.*, 1994; Park *et al.*, 1994; Niewolik *et al.*, 1995). However, most of these studies have employed assays in which p53 proteins were present in crude cell lysates. A systematic examination of the DNA binding properties of purified tumor-derived mutant p53 proteins has not yet been reported.

Since wild-type p53 is a tumor suppressor and mutant forms cannot suppress tumorigenesis (and may even actively contribute to neoplasia) one goal would be to either destroy cells with mutant p53 or identify means to convert a mutant protein to one with normal wild-type function. With respect to the latter objective the ideal result would be to identify a general means to restore normal p53 function to most, if not all, mutants. DNA binding appears to be essential for normal p53 function, and it follows that examination of the ways in which mutant forms of p53 are defective in this regard is of considerable potential importance. We report here that p53 hot-spot mutants are temperature-sensitive for binding to DNA. Additionally we have identified a means by which all mutants tested can bind

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well to DNA at 37 °C. The possibility of a general approach to stabilizing wild-type function in mutant p53 has therapeutic applications.

MATERIALS AND METHODS

Purification of p53 Proteins—Recombinant baculoviruses expressing wild-type and mutant p53 have been described (Friedman *et al.*, 1990; Bargonetti *et al.*, 1992). Extracts of infected sf21 insect cells were prepared, and p53 was purified from lysates by immunoaffinity procedures (Wang *et al.*, 1989). Purified p53 protein was prepared with protein A-Sepharose columns cross-linked with the p53-specific monoclonal antibody PAb 421 (Harlow *et al.*, 1981). The proteins were eluted either with a molar excess of PAb 421 epitope containing peptide (KKGQSTSRHKK-OH) (Wade-Evans and Jenkins, 1985) or with 50% ethylene glycol. Results were similar when proteins were eluted by either method. The protein was dialyzed into Dialysis Buffer containing 10 mM HEPES (pH 7.5), 5 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol. Our initial experiments were conducted with all but p53(Ser-249) mutant protein, which was not yet available as a recombinant baculovirus. More recently, after successfully constructing this baculovirus, we repeated many of the experiments with the p53(Ser-249) protein and, where possible, have included them as well.

Papain Cleavage of Monoclonal Antibodies—To a solution of PAb 1801 (5 mg/ml) in 100 mM sodium acetate (pH 5.3) was added a 1/20 volume of cysteine from a 1 M stock and a 1/20 volume of EDTA from a 20 mM stock. After addition of 10 μg of papain per mg of antibody, reaction mixtures were incubated for 12 h at 37 °C. Iodoacetamide was then added to a final concentration of 75 mM, and mixtures were incubated for 1 h at room temperature prior to dialysis in phosphate-buffered saline (PBS).[1]

Gel Mobility Shift Assays—EMSA was carried out as described (Peterson *et al.*, 1990). The synthetic double-stranded oligonucleotides used in this study include the following: RGC, 5′ TCGAGTTGCCTGGACTTGCCTGGCCTTGCCTTTTC 3′; mutant RGC, 5′ TCGAGTTTAATGGACTTTAATGGCCTTTAATTTTC 3′; SCS, 5′ TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC 3′; GADD45, 5′ AATTCTCGAGCCCAGCATGCTTAGACATGGTTCTGCTCGAG 3′; and mutant GADD45, 5′ AATTCTCGAGCCCAGAATTCTTAGAAATTGTTCTGCTCGAG 3. The probes were ^{32}P-labeled using the Klenow fragment of *Escherichia coli* DNA polymerase. Binding reaction mixtures contained 20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.25% Nonidet P-40, 2 mM spermidine, 10% glycerol, 0.1 ng of bovine serum albumin, double-stranded poly[d(I-C)] (60 ng), and ^{32}P-labeled oligonucleotide (8 ng). p53 protein concentrations are as indicated in figure legends, and in all cases volumes were equalized with Dialysis Buffer. Mixtures were incubated at the indicated temperatures for 30 min unless stated otherwise. In experiments with added antibodies the final reaction volumes remained at 20 μl. In time course of heating experiments, mixtures were incubated without the DNA probe for 0–10 min at 37 °C; the probe was added, and reactions were incubated for 30 min at 25 °C. In competition experiments unlabeled oligonucleotides were added at indicated concentrations directly to the reaction mixtures, and the binding reaction was continued for 30 min at 25 °C. In all cases mixtures were then loaded onto a native 4% polyacrylamide gel containing 0.5 × Tris borate-EDTA (TBE) buffer, 1 mM EDTA, and 0.05% Nonidet P-40 and electrophoresed in 0.5 × TBE at 4 °C at 180–200 V (not exceeding 40 mA current) for 2 h.

DNA Filter Binding—Reaction mixtures (20 μl) containing 20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 1 μl of 40 mM spermidine, 1 μl of 10 mM dithiothreitol, 1 μl of 0.5% Nonidet P-40, 1 μl of 60 μg/ml double-stranded poly[d(I-C)], 1 μl of 2 mg/ml bovine serum albumin, 4 ng of ^{32}P-labeled wild-type or mutant GADD45, and p53 protein were incubated for 30 min at 25 °C. Reaction mixtures were filtered through 0.45-μm nitrocellulose filters presoaked in 25 mM HEPES (pH 7.9), containing 10 ng/μl double-stranded poly[d(I-C)], washed three times with 25 mM HEPES (pH 7.9), dried, and counted by liquid scintillation.

Transfection Assays—H1299 cells at a density of 6 × 10^5 cells/6-cm dish were transfected by the calcium phosphate method with the p53 expressing pCMVneoBam or parental pCMVneoBam vectors (2 μg) containing wild-type or mutant p53s (Kern *et al.*, 1992) along with the mdm2 promoter reporter construct, pGL2-NA(mdm2)-luc (Juven *et al.*, 1993), which contains the *Nsi*I-*Apa*I fragment of the murine *mdm2* gene cloned into the pGL2-Basic vector (Promega). Six hours after incubation with the precipitate at 37 °C, cells were glycerol-shocked for 1 min (10% glycerol in DMEM) and then washed twice in DMEM before adding RPMI medium containing 10% fetal bovine serum. After incubation for an additional hour at 37 °C, cells were transferred to an incubator maintained at 26 °C and kept there for 42 h prior to extraction and determination of luciferase activity.

RESULTS

DNA Binding by p53 Proteins Is Temperature-sensitive—Our studies were performed with wild-type p53 and five tumor-derived mutant p53 proteins that were immunopurified from recombinant baculovirus-infected insect cells using a column containing the p53 monoclonal antibody PAb 421 (Wang *et al.*, 1989). We estimate that the proteins produced by this protocol are approximately 80% pure as judged by silver-stained protein gels (Fig. 1*a*). The slight differences in electrophoretic mobility of the various p53 proteins may be due to altered structure resulting from the mutation and/or possible polymorphism(s) elsewhere in p53, such as that at codon 72, shown to result in altered migration of p53 polypeptide (Matlashewski *et al.*, 1987).

Using the electrophoretic mobility shift assay (EMSA), we examined DNA binding by p53 proteins at 25 or 37 °C to labeled oligonucleotides containing versions of the following p53 response elements: GADD45, RGC, and SCS (Fig. 1, *b* and *c*). GADD45 (Kastan *et al.*, 1992) and RGC (Kern *et al.*, 1991b) are sites that have been identified in human genomic DNA while SCS contains an optimized p53 consensus sequence derived from Halazonetis *et al.* (1993). At 25 °C all mutants bound to the RGC and GADD45 oligonucleotides, albeit with varying efficiencies, while at 37 °C binding to these sites was either undetectable or extraordinarily weak. Wild-type p53 binding was also decreased at the higher temperature, consistent with recent findings of Hainaut *et al.* (1995) who reported that DNA binding at 37 °C by wild-type p53 synthesized in reticulocyte lysates is 30–40% of that observed at 25 °C. While binding at 25 °C by p53(Ala-143), p53(Trp-248), and p53(His-273) was robust, as quantitated by phosphorimaging, wild-type p53 bound at least 2-fold better than any mutant. DNA binding by either p53(His-175) or p53(Ser-249) mutant p53 proteins at 25 °C was significantly less than the abovementioned three, displaying only ~5 and 10% of wild-type p53 activity, respectively. We frequently observed that fresh preparations of p53(His-175) protein bound very well to DNA but then rapidly lost activity, indicating its conformational instability. By contrast, binding by p53(Ser-249), while initially weak, reproducibly showed no deterioration. Therefore, in many cases further studies were carried out with all mutant proteins except p53(His-175).

To exclude the possibility that the loss of mutant p53 DNA binding at 37 °C was due to the activation of a contaminating protease, Western blotting using three different p53 monoclonal antibodies was performed on DNA binding reaction mixtures that had been incubated at 25 or 37 °C. Equivalent amounts of full-length, immunoreactive wild-type or mutant p53 polypeptides were detected after incubation at the two temperatures, showing that the loss of DNA binding at 37 °C did not result from selective degradation of the mutant p53 proteins (data not shown).

Although each mutant bound well to one or more sites at 25 °C there were some notable differences. In particular, we were surprised to observe that p53(Trp-248) showed no detectable binding to the idealized SCS site at 25 °C, even though p53(His-273) and p53(Ala-143) bound comparably well to this site, and wild-type p53 binds better to SCS than any other

[1] The abbreviations used are: PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; EMSA, electromobility shift assay.

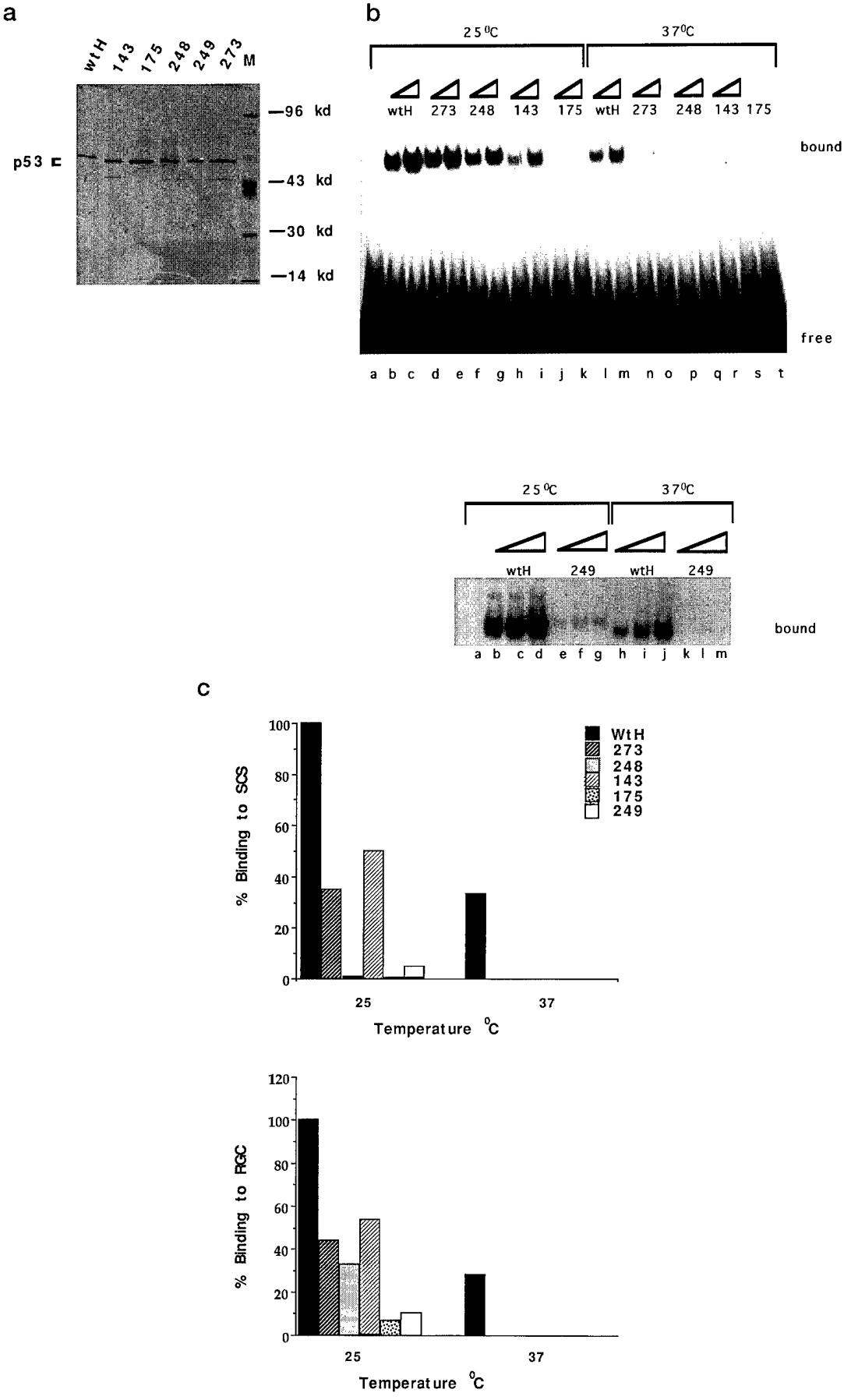

FIG. 1. **Mutant p53 proteins bind DNA at 25 °C.** *a*, p53 proteins. Immunopurified wild-type (*wtH*) and p53(Ala-143), p53(His-175), p53(Trp-248), p53(Ser-249), and p53(His-273) proteins (quantities ranged between approximately 200 and 400 ng) were analyzed by SDS-polyacrylamide

version of the consensus sequence (Halazonetis *et al.*, 1993).[2] p53(His-175) did not bind to SCS either; however, its generally weaker binding to the other sites makes this observation less significant.

There are two critical points to be made from these experiments. 1) All mutants tested have the potential to bind to DNA, and 2) the difference between wild-type and mutant forms of p53 at lower and higher temperatures is such that at physiological temperature wild-type p53 retains significant binding, whereas mutant p53 binding is virtually abolished.

Mutant p53 Proteins Bind DNA in a Sequence-specific Manner—Although the mutant proteins bind well to several DNA sites at lower temperatures, there were concerns that this binding might reflect interactions with DNA that were essentially nonspecific. A nonspecific DNA binding function has been mapped to the C-terminal portion of p53 (Wang *et al.*, 1993), and in the absence of a functional central DNA binding domain, this region alone might be able to bind well to DNA in a sequence-independent manner. Moreover, we previously observed that while the proteolytically excised central "core" domain of wild-type p53 is capable of binding to DNA, the comparable domain released from p53(His-273) protein showed no detectable binding to DNA (Bargonetti *et al.*, 1993). To determine the specificity of DNA binding by mutant p53 proteins, we employed competition EMSA and filter binding assays. Using EMSA it was clear that different unlabeled oligonucleotides containing versions of the wild-type p53 binding site competed for binding to a labeled specific site oligonucleotide far better than did unlabeled oligonucleotides containing mutated binding sites (Fig. 2*a*). Although the amount of unlabeled binding site-containing DNA required for competition varied with the source of p53 and with the competitor, in all cases, at the highest level of competitor tested there were marked differences between the specific and nonspecific sources of DNA (Fig. 2*a*). Strong additional evidence for specificity of mutant p53 interactions with DNA was derived from filter binding experiments (Fig. 2, *b* and *c*) in which efficient binding to wild-type but not mutated p53 binding site oligonucleotides was determined.

Mutant p53 Can Transactivate a p53-responsive Promoter at 26 °C in Vivo—Since the mutant p53 proteins were temperature-sensitive for DNA binding it was of interest to determine whether they have any transcriptional activation capability *in vivo* at lower temperature. Numerous studies have shown that many mutant forms of p53 cannot activate transcription from biologically relevant p53-responsive genes in cells maintained at physiological temperatures (Vogelstein and Kinzler, 1992; Ko and Prives, 1996). We tested whether mutant constructs are able to activate a reporter containing a segment of the murine mdm2 promoter, pGL2-NA(mdm2)-luc (Juven *et al.*,, 1993), following transfection into p53-null H1299 cells at a temperature at which mutant p53 proteins bind *in vitro*. This construct was chosen because we wished to test a biologically relevant p53-responsive promoter. As expected we confirmed that, in contrast to wild-type p53, these mutants were incapable of activating transcription from this promoter at 37 °C (data not shown). In cells transfected at 32 °C we observed that only Ala-143 had transactivation capability, while all other mutants tested were inert (data not shown). That Ala-143 is transcriptionally active at 32 °C is consistent with experiments published by Zhang *et al.* (1994). When the wild-type and mutant p53-expressing constructs were transfected into H1299 cells and then cells were shifted to 26 °C, significant transactivation by three mutant p53 proteins, Ala-143, Trp-248 and His-273, was observed (Fig. 3). Ser-249 and His-175 mutant p53 constructs, however, did not detectably activate transcription over background levels seen with the empty vector. The failure of these latter two mutants to transactivate *in vivo* was not due to reduced expression since Western blotting of transfected cell extracts showed that all p53 constructs tested expressed detectable and roughly comparable levels of p53 protein (data not shown). These transient transfections were performed using a range of plasmid concentrations, and the values shown are the maximal plateau levels. Our data show that some mutant forms of p53 have the intrinsic ability to activate transcription from a physiologically relevant promoter. Those that cannot displayed greater impairment in DNA binding *in vitro*, suggesting a correlation between DNA binding and transactivation.

Mutant p53 Proteins Are Less Thermostable Than Wild-type p53—Since wild-type p53 DNA binding was also somewhat temperature-sensitive, we conducted time course of heating experiments in order to examine the relative thermostability of the p53 proteins (Fig. 4). Wild-type or mutant p53 proteins were incubated in DNA binding buffer at 37 °C for increasing periods; the RGC oligonucleotide was then added, and the reactions were incubated at 25 °C for a further 30 min. While preheating for up to 10 min caused only a 15% loss in DNA binding by wild-type p53, by 7 min of preheating DNA binding by all mutants was reduced to approximately 5% of that seen at 25 °C (Fig. 4). Note that the loss of binding by both the wild-type and mutant proteins was irreversible, as binding was not restored upon the shift to 25 °C.

There were also interesting differences among the mutants in their thermal inactivation properties; p53(Ala-143) and p53(Ser-249) proteins displayed the greatest temperature sensitivity with approximately 80–90% of their binding lost following 2 min of preheating (Fig. 4). By contrast, 2 min of preheating reproducibly actually caused a 2-fold increase in the DNA binding ability of both p53(His-273) and p53(Trp-248) proteins (Fig. 4). This transient stimulation was observed with a wild-type RGC oligonucleotide but not with mtRGC, suggesting that the increased binding is specific (data not shown).

To further understand the thermal sensitivity of p53 proteins, DNA binding over a range of temperatures (25–37 °C) was examined (data not shown). As expected, with increasing temperatures, all p53 proteins showed decreased ability to bind to an oligonucleotide containing the RGC site, although there was a relatively far sharper decline with the mutants than with wild-type form of p53 with the most drastic relative decrease in mutant p53 binding occurring between 33 and 37 °C.

[2] P. Friedlander, Y. Legros, T. Soussi, and C. Prives, unpublished data.

gel electrophoresis. Standard polypeptide markers (*lane M*; molecular mass in kilodaltons) are shown on the *right*. *b, upper panel*, gel mobility shift assays were used to analyze the DNA binding properties of wild-type and mutant p53. The p53 proteins examined include wild-type (*lanes b, c, l,* and *m*), and mutant p53 proteins, p53(His-273) (*lanes d, e, n,* and *o*), p53(Trp-248) (*lanes f, g, p,* and *q*), p53(Ala-143) (*lanes h, i, r,* and *s*), and p53(His-175) (*lanes j, k,* and *t*). Binding to a ^{32}P-labeled GADD45 oligonucleotide (8 ng) was assayed at 25 °C (*lanes b–k*) and 37 °C (*lanes l–t*). The amount of p53 protein added was 0 ng (*lane a*), 200 ng (*lanes b, d, f, h, j, l, n, p,* and *r*), and 400 ng (*lanes c, e, g, i, k, m, o, q, s,* and *t*). *Lower panel*, binding by wild-type (100 ng, *lanes b* and *h*; 200 ng, *lanes c* and *i*; or 400 ng, *lanes d* and *j*) or p53(Ser-249) (100 ng, *lanes e* and *k*; 200 ng *lanes f* and *l*; 400 ng, *lanes g* and *m*) p53 proteins to ^{32}P-labeled GADD45 oligonucleotide (8 ng) was carried out at 25 or 37 °C as indicated. A mixture containing no p53 was run in *lane a*. *c*, DNA binding to ^{32}P-labeled SCS and RGC oligonucleotides (8 ng) was analyzed as described in *b*. Radiolabeled p53·DNA complexes were scanned and quantified using a phosphorimager. The relative levels of binding by 400 ng of p53 are shown graphically with binding by wild-type p53 at 25 °C set at 100%.

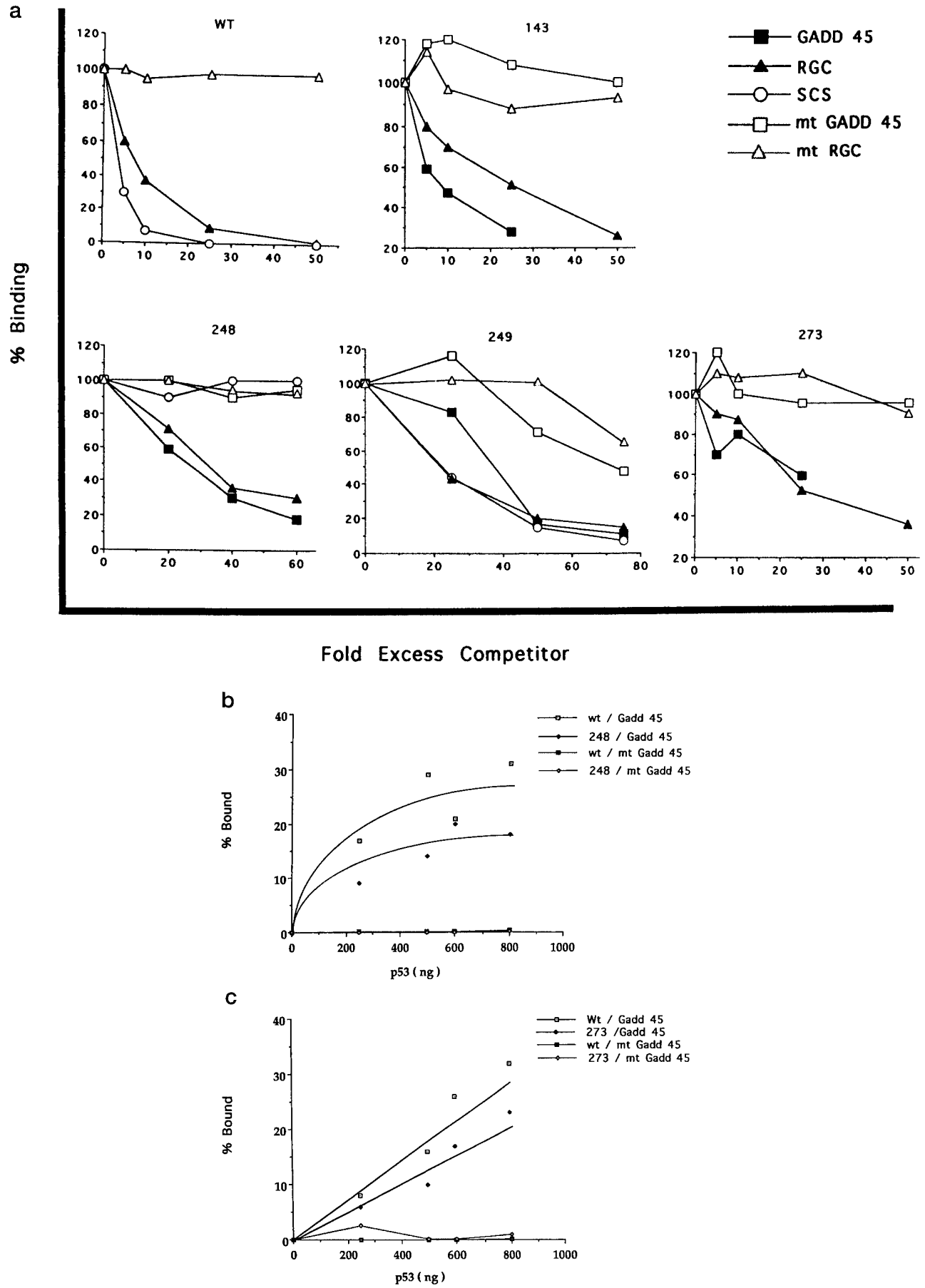

FIG. 2. **Sequence-specific DNA binding by mutant p53 proteins.** *a*, competition EMSA. Wild-type or mutant p53 proteins (200 ng) were bound to ^{32}P-labeled oligonucleotides in the presence of unlabeled oligonucleotides as indicated. Mixtures with wild-type p53, p53(Ala-143), and

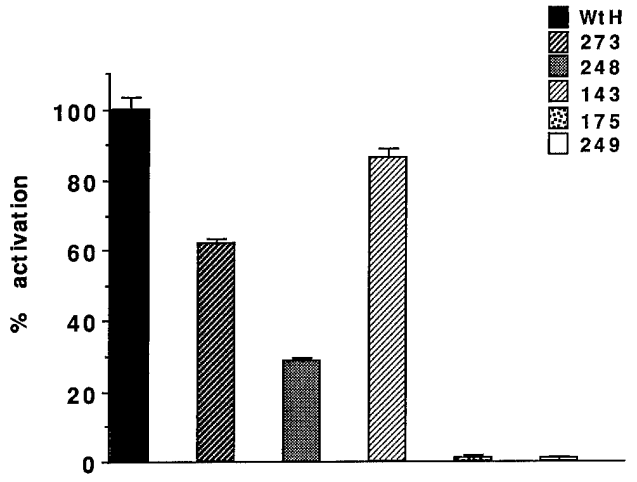

FIG. 3. **Transactivation of a p53-responsive promoter by mutant p53 at 26 °C.** H1299 cells were transfected with constructs expressing wild-type or mutant p53 proteins along with the murine mdm-2 promoter-luciferase reporter plasmid. Luciferase activity was determined in extracts of cells that had been maintained at 26 °C. Values presented are relative induction of luciferase activity over that detected in cells transfected with the parental (empty) pCMVneoBam vector. Induction by wild-type p53, which was 21-fold over background, is set at 100%. Each value represents the average of three samples, and error bars show the standard deviation.

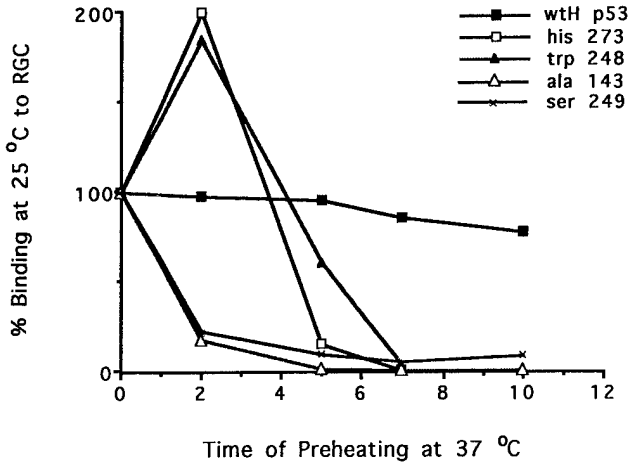

FIG. 4. **Relative heat stability of wild-type and mutant p53 proteins.** Wild-type or mutant p53 proteins as indicated (240 ng) were preincubated at 37 °C for 0, 2, 5, 7, 10 min. Then ^{32}P-labeled RGC oligonucleotide (8 ng) was added and mixtures incubated for 30 min at 25 °C. Radiolabeled p53·DNA complexes were scanned and quantified using a phosphorimager. The relative levels of binding are shown graphically with binding by wild-type or mutant p53 at 0 min of preincubation set at 100%.

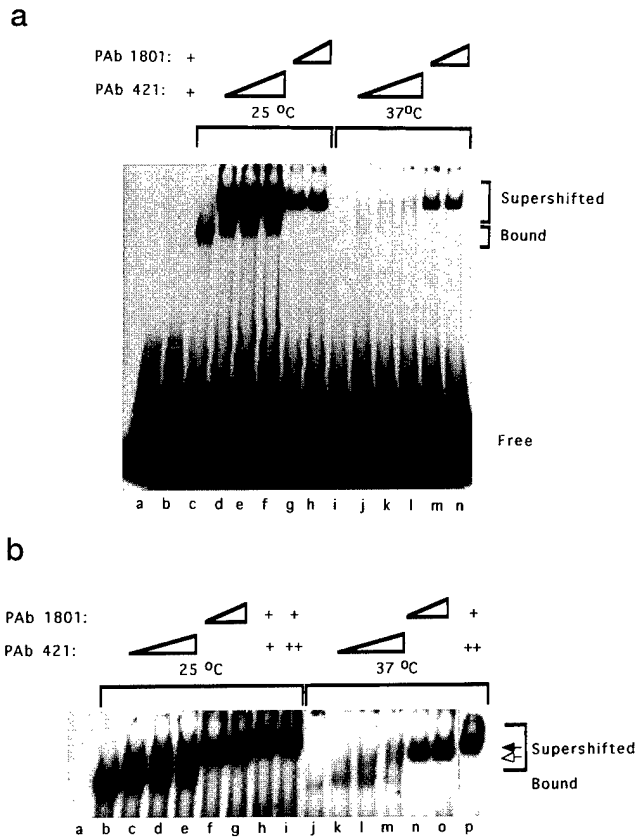

FIG. 5. **Antibody stabilization of DNA binding by mutant p53 proteins.** *a,* DNA binding by p53(Ala-143) (200 ng) to ^{32}P-labeled RGC (8 ng) at 25 °C (*lanes c–h*) and at 37 °C (*lanes i–n*) in the absence of antibody (*lanes c* and *i*) or in the presence of PAb 421 (250 ng, *lanes d* and *j*; 500 ng, *lanes e* and *k*; or 750 ng, *lanes b, f,* and *l*) or of PAb 1801 (250 ng, *lanes g* and *m*; or 500 ng, *lanes b, h,* and *n*). No p53 was present in mixtures run in *lanes a* and *b*. *b,* EMSA analysis of binding by p53(Trp-248) (200 ng) to ^{32}P-labeled GADD45 (8 ng) at 25 °C (*lanes b–i*) and 37 °C (*lanes j–p*) in the absence of antibody (*lanes a, b,* and *j*) or presence of PAb 421 (250 ng, *lanes c* and *k*; 500 ng, *lanes d, h,* and *l*; and 750 ng, *lanes e, i, m,* and *p*) or PAb 1801 (250 ng, *lanes f, h, i, n,* and *p*; and 500 ng, *lanes g* and *o*). No p53 was present in mixture run in *lane a.*

The Monoclonal Antibody PAb 1801 Stabilizes DNA Binding by Wild-type and Mutant p53 at 37 °C—Several studies have documented the regulation of p53 sequence-specific DNA binding by sequences and sites within its C terminus. In particular, the monoclonal antibody PAb 421 (Harlow *et al.,* 1981) that interacts with an epitope (amino acids 373–381) within the C terminus of p53 (Wade-Evans and Jenkins, 1985) can enhance the DNA binding function of wild-type (Hupp *et al.,* 1992; Halazonetis *et al.,* 1993) and even certain mutant forms (Hupp *et al.,* 1993; Halazonetis *et al.,* 1993) of p53. Thus, it was of interest to compare the effect of PAb 421 on mutant p53 binding at lower and physiological temperatures. p53 proteins were incubated with oligonucleotides containing p53 binding sites at 25 or 37 °C in the presence or absence of PAb 421. As a control, we used an antibody that recognizes an epitope (amino acids 46–55) within the N terminus of p53, PAb 1801 (Banks *et al.,* 1986; Legros *et al.,* 1994). PAb 421 increased DNA binding by mutant p53 proteins at 25 °C (Fig. 5). However, binding in the presence of PAb 421 was significantly reduced at 37 °C. Additionally, DNA binding by wild-type p53 synthesized *in vitro* in reticulocyte extracts was stimulated by PAb 421 at 25 °C but rather poorly at 37 °C.[2] These experiments therefore show that while PAb 421 increases the DNA binding function of wild-type and mutant p53 proteins at 25 °C, this stimulation is reduced at 37 °C and is thus temperature-sensitive. It should be noted, however, that at 37 °C there was detectable binding in the

p53(His-273) contained ^{32}P-labeled RGC oligonucleotide as probe, while mixtures with p53(Ser-249) or p53(Trp-248) contained ^{32}P-labeled GADD45 oligonucleotide as probe. Electromobility shift assays were performed and the DNA-protein complexes quantitated using a phosphorimager. Binding with no competitor was set at 100%. *b* and *c,* filter binding analysis. Wild-type and p53(Trp-248) p53 proteins (*b*) or wild-type and p53(His-273) p53 proteins (*c*) were bound to ^{32}P-labeled GADD45 oligonucleotides (4 ng) and passed through nitrocellulose filters. Protein-DNA complexes were quantitated by scintillation counting, and the percentage of the input DNA bound to the filter was determined (% bound).

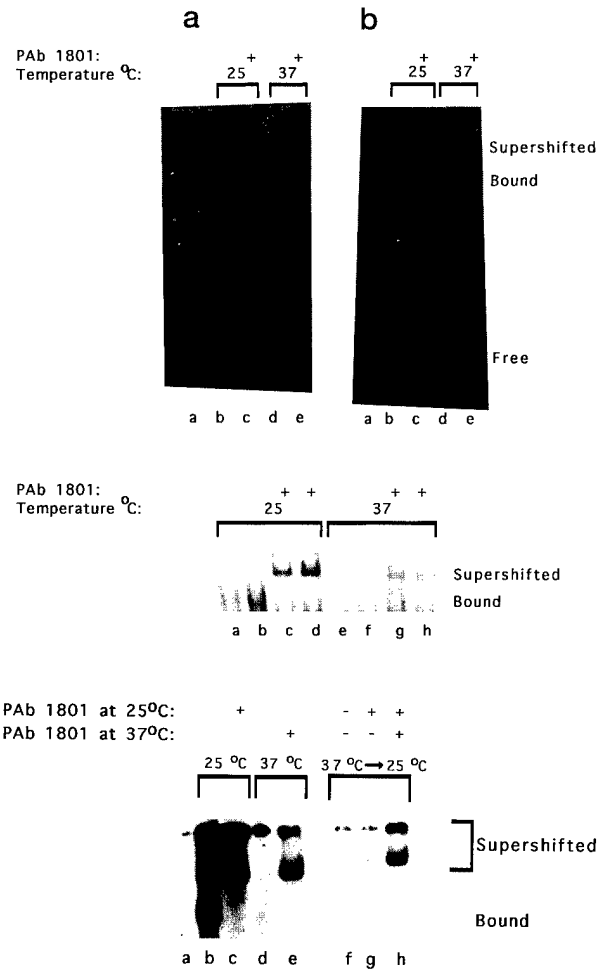

FIG. 6. **PAb 1801 stabilizes but does not restore DNA binding by mutant p53 protein at 37 °C.** *a* and *b*, gel mobility shift assays were performed to determine binding by p53(Trp-248) to ^{32}P-labeled RGC (8 ng) (*a*) or by p53(His-175) to ^{32}P-labeled GADD45 oligonucleotide (8 ng) (*b*) at 25 °C (*lanes b* and *c*) and 37 °C (*lanes d* and *e*). Binding was examined in the absence (*lanes a, b,* and *d*) and presence (*lanes c* and *e*) of purified monoclonal antibody PAb 1801 (500 ng). *Lane a* contains no p53 protein. 200 ng of mutant p53 proteins were used. *c*, EMSA was performed to determine binding by p53(Ser-249) (200 ng, *lanes a, c, e,* and *g*; or 400 ng, *lanes b, d, f,* and *h*) to ^{32}P-labeled GADD45 oligonucleotide (8 ng) at 25 or 37 °C as indicated. Binding was examined in the absence (*lanes a, b, e,* and *f*) or presence (*lanes c, d, g,* and *h*) of PAb 1801 (600 ng). *d*, mixtures containing p53(Ala-143) (200 ng) were incubated either at 25 °C for 30 min (*lanes b* and *c*), 37 °C for 30 min (*lanes d* and *e*), or for 30 min at 37 °C followed by 30 min at 25 °C (*lanes f, g,* and *h*). PAb 1801 was present in mixtures run in *lanes c, e, g,* and *h*. In *lane g* the antibody was added following the 30 min at 37 °C, while in *lane h* the antibody was present prior to the 30 min at 37 °C.

presence of PAb 421, which was greater than that seen in its absence.

Surprisingly, in contrast to the results obtained with PAb 421 at 37 °C, the control antibody PAb 1801 showed strong rescue of mutant p53 binding at 37 °C (Fig. 5, *a* and *b*). Although very little stimulation of p53 binding to DNA by PAb 1801 was observed at 25 °C, this monoclonal antibody prevented loss of binding at 37 °C by all mutants tested (Figs. 5 and 6).

We asked whether PAb 421 is capable of stimulating p53 DNA binding in the presence of PAb 1801 at 37 °C by analyzing DNA bound by mutant p53 incubated with both PAb 1801 and PAb 421. We observed DNA binding at 37 °C comparable or slightly greater in amount to that seen when PAb 1801 alone was added (Fig. 5*b*, *lanes n–p*, and data not shown). The fact that the DNA protein complex migrated more slowly when both antibodies were used suggests that the PAb 1801-stabilized p53·DNA complexes still express the PAb 421 epitope and also that PAb 421 retains an affinity for p53 at 37 °C (Fig. 5*b*, *lanes h, i,* and *p*). Thus, the reduced ability of PAb 421 to rescue binding by p53 at 37 °C is not simply due to a lessened ability by the antibody to recognize the p53 protein at the higher temperature.

Stabilization in the presence of PAb 1801 was achieved for each mutant with each of the three different binding sites tested (Fig. 6 and data not shown). Indeed, even binding by p53(His-175), which, as expected, bound to GADD45 with markedly lower affinity than the other mutant proteins tested at 25 °C, was stabilized in the presence of PAb 1801 (Fig. 6*b* compare *lanes d* and *e*). Additionally, PAb 1801, but not PAb 421, secured DNA binding of reticulocyte lysate expressed wild-type p53 at 37 °C (data not shown). Furthermore, the addition of SF21 cell extract to gel shift reaction mixtures containing purified mutant p53 protein did not alter 1) the temperature sensitivity of the p53 mutants, 2) the ability of PAb 1801 to thermostabilize DNA binding, and 3) the ability of PAb 421 to stimulate DNA binding in a temperature-sensitive manner. In summary, all of the data we have obtained with PAb 1801 suggest that this antibody prevents loss of binding by each mutant at physiological temperature but does not qualitatively change its intrinsic binding ability. For example, PAb 1801 did not allow p53(Trp-248) to bind SCS (data not shown). Moreover, this antibody did not stabilize binding by mutant p53 proteins to a mutated RGC sequence (data not shown).

To determine if DNA binding that was secured by PAb 1801 is dependent upon the presence of DNA, we incubated p53 at 37 °C in DNA binding buffer (lacking DNA) with PAb 1801. Then the RGC oligonucleotide was added and the reactions incubated at 25 °C for 30 min. Comparable binding to RGC by p53(Ala-143), p53(His-273), and p53(Trp-248) was seen in the reaction mixtures containing PAb 1801 to what was detected when DNA was present from the start of the reaction as in the normal protocol (data not shown). This demonstrates that the ability of PAb 1801 to stabilize p53 proteins in a DNA binding positive conformation does not depend upon the presence of a p53 DNA binding site at 37 °C.

PAb 1801 might affect binding at 37 °C either by maintaining the p53 protein in a DNA binding positive conformation or by restoring such a conformation to DNA binding negative protein. To differentiate between these possibilities, we incubated mutant p53 with RGC or SCS at 37 °C in the presence or absence of PAb 1801 (Fig. 6*d*). Then PAb 1801 was added to those samples lacking PAb 1801, and the incubation was continued at 25 °C. p53 proteins bound to DNA only when PAb 1801 was present throughout the 37 °C incubation period. A typical example is shown for p53(Ala-143) binding to SCS (Fig. 6*d* compare *lane h* to *lanes f* and *g*), and this result held for the other mutants binding to SCS as well as to RGC (data not shown). Thus, PAb 1801 allows the mutant proteins to remain in a conformation capable of binding DNA, as opposed to restoring such conformation to thermally inactivated p53 protein.

The difference in the relative abilities of PAb 1801 and PAb 421 to prevent loss of mutant p53 binding at 37 °C led us to examine other antibodies recognizing either the same or different epitopes within p53. The immunogenicity of p53 has been studied in some detail previously. Both the N- and C-terminal regions are markedly immunodominant, and within these regions are a number of epitopes that have repeatedly elicited antibodies both in experimental models (Legros *et al.*, 1994) and in human patients (*e.g.* Schlichtholz *et al.*, 1992; Schlichtholz *et al.*, 1994). We evaluated the ability of a number of

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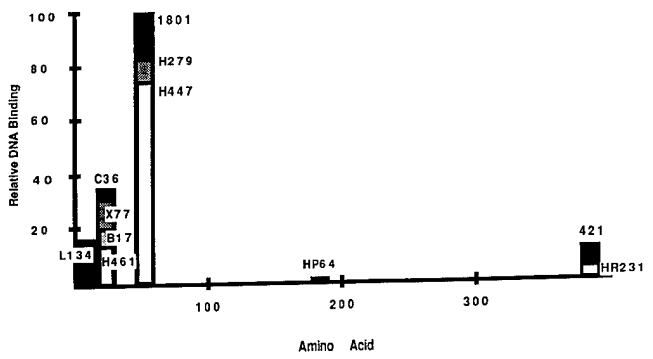


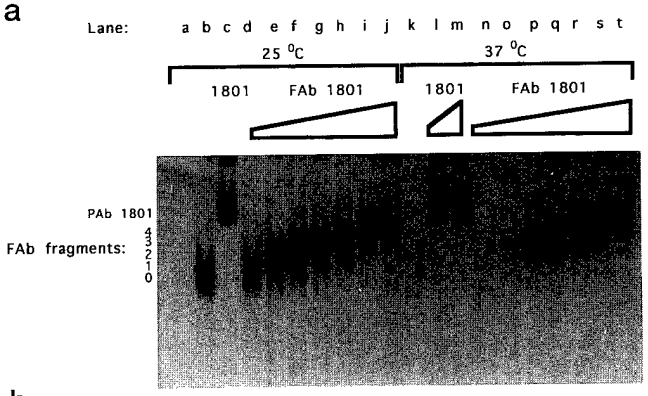
b

Relative DNA Binding

Epitope	Antibody	Mutant p53 protein 143	248	249	273
11-20	L134	17	18	5	20
16-30	C36	36	45	38	39
	B17	19	15	37	14
	H461	14	17	14	12
46-55	1801	100	100	100	100
	H447	76	84	58	87
	H279	83	52	49	64
171-185	HP64	1	1	1	2
371-380	421	12	20	8	18
	HR231	4	1	4	-

FIG. 7. **Survey of antibodies affecting mutant p53 DNA binding at 37 °C.** *a*, gel mobility shift assays were performed to determine the relative ability of various antibodies to stabilize binding by p53(Ala-143) to ^{32}P-labeled GADD45 oligonucleotide (8 ng) at 37 °C. 200 ng of p53 protein was used. Binding was determined over a range of antibody concentrations (up to 750 ng), and the maximal protein-DNA complexes obtained were quantitated using a phosphorimager. We used the values obtained for plateau levels of binding. Maximal binding in the presence of PAb 1801 was set at 100%, and the ability of the various antibodies to stabilize DNA binding were plotted relative to that value. The antibodies included L134 (epitope: amino acids 11–20) as ascitic fluid from mouse diluted 10-fold in PBS; B17, C36, and H461 (epitope: amino acids 16–30), PAb 1801, H279, and H447 (epitope: amino acids 46–55), HP64 (epitope: amino acids 171–185), PAb 421 (epitope: amino acids 370–378), and HR 231 (amino acids 371–380) all in PBS; and X77 (epitope: amino acids 16–25) in DMEM + 10% fetal calf serum. *b*, tabular representation of the relative ability of antibodies discussed in *a* to stabilize DNA binding by p53(Ala-143), p53(His-273), p53(Trp-248), and p53(Ser-249) at 37 °C. Stabilization byPAb 1801 was set at 100%.

a



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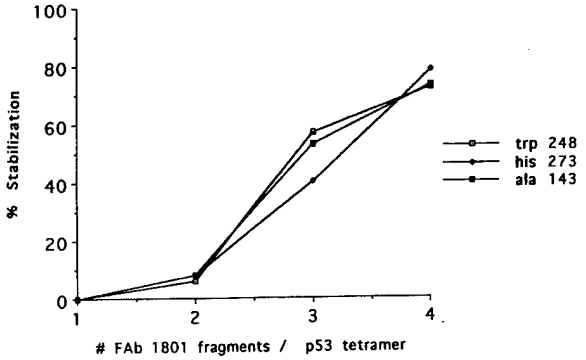

FIG. 8. **Stabilization of DNA binding by monovalent FAb 1801 fragments.** *a*, p53(Trp-248) protein (350 ng) was bound to ^{32}P-labeled GADD45 oligonucleotide (8 ng) at 25 °C (*lanes a–j* in *a*) or 37 °C (*lanes k–t* in *a*). *a*, PAb 1801 was added to the mixtures run in *lane l* (500 ng) and *lanes c* and *m* (800 ng). FAb 1801 was added to the mixtures run in *lanes d* and *n* (66 ng), *lanes e* and *o* (200 ng), *lanes f* and *p* (350 ng), *lanes g* and *q* (470 ng), *lanes h* and *r* (660 ng), *lanes i* and *s* (900 ng), or *lanes j* and *t* (1.1 μg). Mixtures run in *lanes b* and *k* had no antibody, and mixture run in *lane a* had no p53. *c*, graphical representation of stabilization of the DNA binding by mutant p53 proteins at 37 °C. Stabilization of FAb 1801 fragments is relative to that obtained when PAb 1801 was used as determined through phosphorimaging.

purified monoclonal antibodies recognizing epitopes within amino acids 11–20, 16–25, 16–30, 46–55, 171–185, or 373–381 to stabilize the DNA binding function of p53. Fig. 7 shows our results expressed as maximal affect on DNA binding at 37 °C by a given antibody (previously determined after testing a range of concentrations of each antibody) normalized to the maximal binding in the presence of PAb 1801. We first determined that all the antibodies supershifted and did not inhibit the formation of p53·DNA complexes at 25 °C.[2] Importantly, each of the mutants studied revealed essentially similar patterns of stabilization by the different antibodies. Two antibodies, H279 and H447, each recognizing a similar epitope to PAb 1801, were clearly the most effective, although neither was quite as impressive as PAb 1801, and the two varied in their relative ability to affect binding of different mutant proteins. Antibodies recognizing amino acids 16–30, particularly C36, also contributed significantly to p53 binding at 37 °C (12–45%), although to lesser extents than did the 46–55 series. An antibody to the epitope within amino acids 11–20 allowed for even less stabilization (5–20%). The antibody PAb HP64 that recognizes a cryptic epitope in the central portion of the p53 protein had no effect whatsoever. Finally, both PAb 421 and another monoclonal antibody, HR231, which each recognize an epitope within amino acids 371–380, provided only a small amount of stabilization of DNA binding at 37 °C (1–18%) even though both antibodies greatly stimulated DNA binding by 10–25-fold at 25 °C. Thus, antibodies recognizing the region spanning amino acids 46–55 are the most effective in stabilizing DNA binding by mutant p53.

FAb Fragments of PAb 1801 Stabilize Mutant p53 DNA Binding—The effect on DNA binding by N-terminal antibodies was conceivably the result of cross-linking of the p53 tetramer. To address this possibility, we generated FAb fragments of PAb 1801 (FAb 1801) and determined their ability to stabilize DNA binding by p53 (Fig. 8, *a* and *b*). Monovalent FAb 1801 fragments clearly allowed binding by all mutants tested at 37 °C. The slightly lower degree of stabilization by FAb 1801 *versus* PAb 1801 might result from a lower affinity for p53 or from cross-linking acting as an additional but not essential component of the effect of PAb 1801. Interestingly, we observed that the presence of three or four FAb fragments per p53 tetramer allowed significantly greater binding than did one or two FAb fragments per tetramer. This suggests that maximal stabiliza-

tion of mutant p53 binding requires interaction with all four of the monomers in a p53 tetramer but that the bivalent form of an antibody (*i.e.* one capable of cross-linking monomers) is not necessary for rescue of mutant p53 DNA binding from thermal inactivation.

DISCUSSION

DNA binding is very likely to be an important function of wild-type p53. The rather extraordinary clustering of tumor-derived mutations within the DNA binding domain of p53 strongly suggests that this region is critical for the normal role of p53 in tumor suppression. Reciprocally, the fact that mutations are focused in this region leads to the assumption that alteration of residues in this region would destroy or alter DNA binding. We have demonstrated, however, that hot-spot mutant p53 proteins are inherently capable of binding specifically to DNA. Binding varies somewhat with the mutation and the version of the consensus site used and occurs only at sub-physiological temperatures. Additionally, we have identified a means by which temperature-sensitive binding by mutant p53 can be stabilized such that significant levels of binding can be obtained at physiological temperatures.

The co-crystal structure of the central p53 DNA binding domain bound to DNA (Cho *et al.*, 1994) has been most informative. Aside from providing the three-dimensional positions of the different amino acids that make up the domain and their relationship to a cognate p53 DNA site, the structure has provided insight into the role of the amino acids that are most frequently mutated in human cancer in binding to DNA. It is possible to divide p53 tumor-derived hot-spot mutations into two classes, those that affect directly the interaction between protein side chains and DNA and those that affect the stable conformation of the domain (reviewed by Cho *et al.*, 1994). Two that contact the DNA directly are Arg-248, in the minor groove, and Arg-273 at a backbone phosphate. However, these are two out of several amino acids that make direct contact with DNA. It is interesting that the other contact residues are not mutated with unusual frequency. Rather, the other four hot spots are involved in maintaining the structure of the DNA binding motifs. The two classes of hot-spot mutants, contact and conformational, can be differentiated by other criteria as well; these include binding to heat shock cognate protein Hsc70 (Hinds *et al.*, 1990), recognition by the conformation-specific antibody PAb 240 (Gannon *et al.*, 1990), ability to function as a transcriptional activator when fused to a GAL4 DNA binding domain (Raycroft *et al.*, 1991; Unger *et al.*, 1992), and protease sensitivity (Bargonetti *et al.*, 1993). The two classes of mutants might be expected to display significant variation in their interactions with DNA, and certainly differences among the mutants exist both in efficiency and specificity of binding. Nevertheless, given these differences it is extraordinary that all mutants tested show DNA binding capability and that all are temperature-sensitive. Possibly, under less stringent conditions, the very size and complexity of the p53 DNA binding domain allows for some degree of interaction with DNA by p53 gene products that have sustained a single mutation within this region.

One might view our results as follows: each missense mutation uniquely affects specific DNA or amino acid contacts or alters the over-all conformation of the DNA binding domain. This results in the varying degrees to which different mutants are impaired for binding to DNA, as well as some of the relative differences seen with different DNA binding sites. Any p53 protein, however, whether wild-type or mutant is sensitive to thermal stress at 37 °C. The combination of lower affinity DNA binding and thermal lability would result in no observable binding by mutant proteins at 37 °C. The temperature-sensitive phenotype of certain p53 mutants in cells has been observed previously. Both murine p53(Val-135) (Michalovitch *et al.*, 1990; Martinez *et al.*, 1991) and human p53(Ala-143) (Zhang *et al.*, 1994) display a temperature-conditional nature in cells. Chimeric polypeptides containing the GAL4 DNA binding domain fused with mutant p53 have also displayed temperature sensitivity for transcriptional activation (Unger *et al.*, 1992). Indeed, a number of studies have documented DNA binding by mutant p53 in cell extracts, suggesting that even at physiological temperatures some mutants might have partial wild-type function (Chen *et al.*, 1993; Chumakov *et al.*, 1993; Miller *et al.*, 1993; Zhang *et al.*, 1993; Park *et al.*, 1994; Niewolik *et al.*, 1995). However, it is clear from comparing these reports that the cell environment can affect whether and how various p53 mutants display DNA binding and transcriptional activation. Unfortunately, it is also frequently the case that mutants are "functional" in cells only when idealized consensus sites are used for DNA binding or as transcriptional response elements in reporter constructs. Our data show that purified mutant p53 proteins can bind to a variety of sites including ones from physiologically relevant genes. The ultimate goal will be to restore full wild-type p53 function to mutant forms of the protein in tumor cells.

The complexity and size of the DNA binding domain and the fact that it alone does not contain known regulatory sequences has led to the suggestion that it may be difficult to identify small molecules that interact directly with the DNA binding domain to restore function to defective mutants (Friend, 1994). However, there are several lines of evidence that sequences and signals outside of the DNA binding region can be propagated to affect the functioning of the DNA binding domain. Most of these are within the C-terminal portion of p53. Within the last 30 amino acids is a highly basic region that when bound by antibody or bacterial heat shock protein, dnaK, leads to marked activation of specific DNA binding (Hupp *et al.*, 1992). Additionally, phosphorylation of p53 by casein kinase II (Hupp *et al.*, 1992), protein kinase C (Delphin and Baudier, 1994), and G_2/S cyclin-dependent kinases (Wang and Prives, 1994) stimulates and alters (Wang and Prives, 1995) p53 sequence-specific DNA binding. Moreover, short DNA single strands stimulate DNA binding by the p53 central domain in a manner that is dependent on the C terminus (Jayaraman and Prives, 1995). Importantly, p53 from which the C-terminal 30 amino acids is deleted binds much better to DNA than full-length p53 (Huppet *et al.*, 1992; Halazonetis *et al.*, 1993). These data taken together suggest that p53 exists in DNA binding negative and positive conformations. The relationship between these two conformations holds for some mutant forms of p53 as well. Indeed, the observation of Bargonetti *et al.* (1993), who showed that a 27-kDa protease-resistant fragment spanning the central DNA binding domain from wild-type but not from p53(His-273) mutant p53 is capable of binding specifically to DNA at 25 °C, supports the likelihood that sequences outside of the DNA binding domain play critical roles in mutant p53 sequence-specific DNA binding. It was therefore disappointing that the powerful stimulation of both wild-type and mutant p53 DNA binding by the C-terminal specific monoclonal antibody, PAb 421, is temperature-sensitive. Our data showing that both PAb 421 and 1801 were able to bind together to mutant p53 at 37 °C are encouraging and provide the possibility that molecules reacting with both N and C termini of p53 might be developed to cooperate in restoring DNA binding to mutant p53.

Recent reports have provided evidence that p53 transcriptional activity is increased in cells microinjected with the monoclonal antibody, PAb 421 (Abarzua *et al.*, 1995; Hupp *et al.*,

1995). These data are consistent with earlier reports showing that injection of a p53 C-terminal specific antibody into cycling cells led to growth arrest (Mercer *et al.*, 1982). Although it was suggested that the increased p53 transcriptional activation resulted from the conversion of p53 from a latent form to one that is active for DNA binding, as occurs *in vitro*, the effect may also have been due to stabilization of p53 protein in cells that were microinjected with PAb 421. Indeed, since wild-type p53 DNA binding is of a highly cooperative nature, a relatively small increase in p53 protein levels might produce a marked increase in DNA binding-dependent transcriptional activation.

The universality of the stabilizing influence by PAb 1801 was unexpected. It was also unanticipated that rescue of the temperature-sensitive phenotype would involve interactions with the N rather than the C terminus of p53. The p53 N terminus, containing the transcriptional activating region, is highly sensitive to protease cutting and thus solvent-exposed (Pavletich *et al.*, 1993). It is also very immunodominant such that the majority of p53 monoclonal antibodies that have been isolated recognize epitopes within the N terminus. Perhaps the flexibility of this region is also responsible for its temperature sensitivity. Transient contacts with the "floppy" N terminus may be deleterious to the functioning of the central DNA binding domain. Such contacts would be more frequent at higher temperatures where random motion would be more recurrent. Interaction with PAb 1801 and related antibodies may reduce negative effects by reducing or eliminating these putative random contacts. Indeed, this region of the protein may well be more accessible to reagents that imitate the action of PAb 1801 type antibodies. It is also a possibility that the antibody is stabilizing conformational changes at a distance. Such effects have been observed with *Drosophila* heat shock factor (Zimarino *et al.*, 1990) as well as the insulin receptor (Roth *et al.*, 1983).

The p53 status of tumor cells is likely to be an important indicator of outcome of therapy (discussed in Lowe *et al.*, 1994). There is compelling evidence that most types of cells cannot tolerate high levels of wild-type p53 and that in many cases, when levels of wild-type p53 are increased, cells die or permanently arrest. Tumor cells frequently express high levels of mutant p53. The possible role that mutant p53 plays in tumor progression is not completely understood, although in the massive p53 literature there are many examples documenting a high correlation between mutant p53 status and poor prognosis. It would seem beneficial to be able to induce tumor cells to express p53 protein with wild-type function. The fact that those tumors in which p53 is wild type such as neuroblastomas, testicular cancers, and lymphoid leukemias have the highest rate of long term survival after radiation treatment is strongly suggestive of a role for wild-type p53 in tumor cell arrest and or death. We have shown that several of the hot-spot mutant forms of p53 can bind specifically to several versions of the p53 consensus sequences, and a subset of these can activate transcription from a p53-responsive promoter at 26 °C. Furthermore, although such binding is highly sensitive to increases in temperature, loss of binding at 37 °C can be curtailed by antibodies that react with epitopes within residues 46–55. Although mutant p53 is synthesized in tumor cells at 37 °C, it is likely that molecules capable of functioning like PAb 1801 would stabilize the wild-type conformation of newly synthesized p53 polypeptides. Our hope is that therapeutic agents can be developed, mimicking the activity of these antibodies, which can restore wild-type function to mutant p53 in tumor cells.

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p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells

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p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells

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It is well established that induction of the p53 tumor suppressor protein in cells can lead to either cell cycle arrest or apoptosis. To further understand features of p53 that contribute to these cell responses several p53-null Saos2 and H1299 cell lines were generated that express wild-type or mutant forms of p53, or the cyclin-dependent kinase inhibitor p21/WAF1, under a tetracycline-regulated promoter. Our results show that the cellular level of p53 can dictate the response of the cell such that lower levels of p53 result in arrest whereas higher levels result in apoptosis; nevertheless, DNA damage can heighten the apoptotic response to p53 without altering the protein level of p53 in cells. We also demonstrate that arrest and apoptosis are two genetically separable functions of p53 because a transcriptionally incompetent p53 can induce apoptosis but not arrest, whereas induction of p21/WAF1, which is a major transcriptional target of p53, can induce arrest but not apoptosis. Finally, we show that a full apoptotic response to p53 requires both its amino and carboxyl terminus, and our data suggest that there is synergism between transcription-dependent and -independent functions of p53 in apoptosis. Thus, there are multiple independent cellular responses to p53 that together may account for the extraordinarily high frequency of p53 mutations in diverse types of human tumors. The implications of these results are discussed and a model is proposed.

[*Key Words*: p53; p21/WAF1; cell-cycle arrest; apoptosis; DNA damage]

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Following genotoxic stress such as that emanating from damaged DNA or conditions of hypoxia, induction of p53 results in cell-cycle arrest or apoptosis (for review, see Levine 1993; Gottlieb and Oren 1996; Ko and Prives 1996). It is not fully understood which of these two responses to p53 is chosen in a given cell. Cell type appears to be one of the factors involved in this process (for review, see Gottlieb and Oren 1996) and the microenvironment of a cell can also influence the response (Boudreau et al. 1995, 1996). In addition, several cellular and viral proteins contribute to the propensity of cells to undergo either response (for review, see Fisher 1994; White 1996).

It is also not fully understood what functions of p53 are required for cell cycle arrest or apoptosis. p53 is a sequence-specific transcriptional activator of genes containing p53 response elements (for review, see Vogelstein and Kinzler 1992). A number of transcriptional targets of p53 have been identified. One of these is the cyclin-dependent kinase inhibitor p21 (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993), which inhibits the protein kinase activities of G_1 cyclin/CDK complexes, thereby preventing phosphorylation of the retinoblastoma (RB) protein (Slobos et al. 1994). p21 is thus an excellent candidate for mediating p53-induced cell-cycle arrest. It is not known whether activation of additional targets of p53 is required for arrest as well.

It is less clear how p53 induces apoptosis. There are, however, at least two candidate genes that play roles in apoptosis that can be transactivated in response to p53 induction. In murine cells, p53 upregulates expression of the *bax* gene (Miyashita and Reed 1995), whose product dimerizes with Bcl-2 and prevents the ability of Bcl-2 to block apoptosis (Oltvai et al. 1993). It is possible, therefore, that transcriptional activation of the *bax* gene by p53 induces apoptosis. A second p53 target that might influence apoptosis is IGFBP-3 (Buckbinder et al. 1995), which is an antagonist of insulin-like growth factor-1 (IGF-1) (Baserga 1994). Down-regulation of IGF-1 or the IGF-1 receptor is correlated with the apoptotic response (Baserga 1994). Several recent studies have provided evidence, however, that p53 may have a transcription-independent function in apoptosis (Caelles et al. 1994;

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Wagner et al. 1994). Furthermore, different reports have provided essentially contradictory results as to the requirement for the sequence-specific transactivation function of p53 for induction of apoptosis (Haupt et al. 1995; Sabbitini et al. 1995). If indeed there is an alternative function of p53, then it will be important to identify the domains or regions of the protein responsible for this function.

p53 has been subjected to extensive analysis of its functional domains (Gottlieb and Oren 1996; Ko and Prives 1996). The p53 polypeptide consists of an activation domain located within the amino-terminal 43 amino acids, a sequence-specific DNA binding domain located within the central, conserved portion of the protein, and, within the carboxyl terminus resides a tetramerization domain as well as a regulatory region that controls the ability of the protein to allosterically switch from a latent form to one that is active for sequence-specific DNA binding. Either naturally occurring or experimentally produced mutations within these regions of p53 have identified residues that are important for their function. Mutation of two residues within the p53 activation region, leu22 and trp23, abrogates transactivation by p53 (Lin et al. 1994). This is presumably the case because these residues are required for the interaction of the activation domain with TATA-box binding protein associated factors (TAFs) (Lu and Levine 1995; Thut et al. 1995). The vast majority of the missense mutations that have been detected in tumors of cancer patients map to the central DNA-binding domain of p53 (Hollstein et al. 1991). Among these are a number of mutational hot spots that occur with unusually high frequency and together make up ~40% of all tumor-derived p53 mutations. Most mutations within the central DNA-binding domain impair or abolish sequence-specific DNA binding by p53 (Vogelstein and Kinzler 1992). Finally, mutational analysis of the carboxyl terminus has revealed residues that are important for oligomerization of the p53 protein (Sturzbecher et al. 1992). Moreover, deletion of the carboxy-terminal 30 amino acids of p53 has striking stimulatory effects on the ability of the protein to bind to DNA in vitro (Hupp et al. 1992; Halazonetis and Kandil 1993).

To further understand how p53 might regulate arrest versus apoptosis we have used the p53 null cell lines Saos2 and H1299 to generate tetracycline-regulated cell lines inducibly expressing either wild-type or mutant forms of p53 or wild-type p21. This system has been utilized previously by others for a variety of goals (Buckbinder et al. 1994; Van Meir et al. 1994; Agarwal et al. 1995; Chen et al. 1995). The lines we have generated have allowed us to (1) identify a quantitative response to different amounts of p53, (2) examine a number of p53 variants in clonally derived cell populations to derive information about the influence of functional domains of p53 on cell arrest and apoptosis, and (3) determine the effect of DNA damage on p53 in this context. Our results have provided new insight into p53 and should provide a paradigm for this approach to studying p53 structure and function.

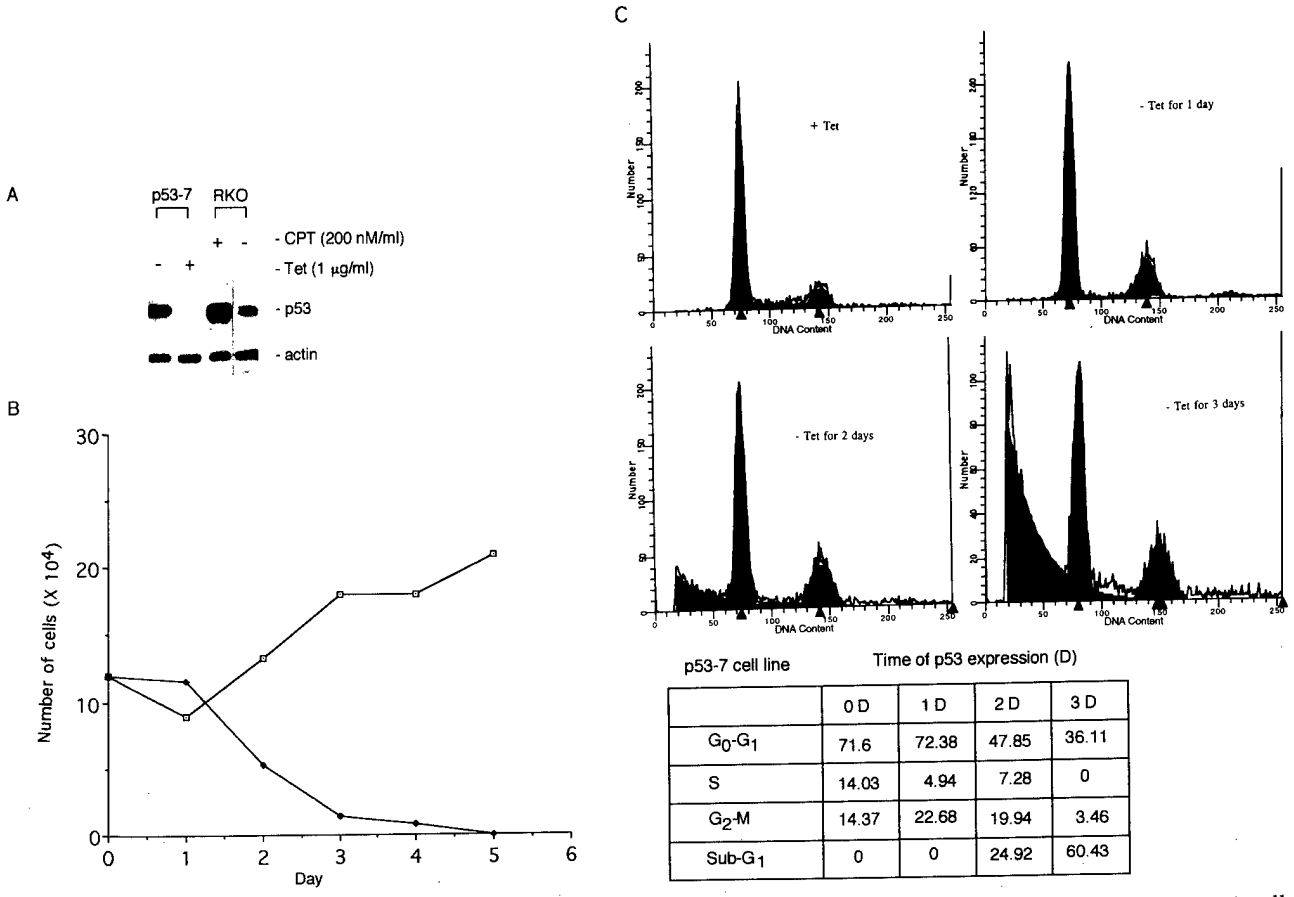
Results

The level of p53 within Saos2 cells determines cell death or arrest

To generate the first series of inducible cell lines, we chose the human osterosarcoma cell line Saos2 because they are null for p53, are easily transfected, and have been shown previously to arrest upon overexpression of wild-type p53 (Chen et al. 1990). Each individual cell line used for this study was chosen from a number of clonal lines based on relative levels of protein expressed after induction.

Of the two wild-type p53 inducible cell lines obtained, one (p53-7) expressed p53 protein at relatively high levels upon withdrawal of tetracycline and will be referred to as the "high p53 producer" line (Fig. 1A). p53 protein was detected within 8 hr and reached maximal levels at 24 hr post-induction (data not shown). Note that the amount of detectable induced p53 protein in p53-7 cells, however, was substantively less than that induced in DNA damaged human RKO cells that contain wild-type p53 (Kastan et al. 1992), demonstrating that the level of p53 expressed in the Saos2 cell line is within the physiological range. When the growth curve of induced and uninduced p53-7 cells was examined, there was a dramatic difference in cell viability between the two states (Figure 1B). The uninduced cells continued to grow with a doubling time of ~48 hr, whereas the p53 expressing cells started to die within 2 days (as determined by reduced cell count), and by 3 days, only 10% of the cells survived. By 5 days after induction there were virtually no visible cells remaining on the plate (data not shown). DNA histogram analysis of induced p53-7 cells (Fig. 1C) showed that 1 day after tetracycline withdrawal the percentage of cells in S phase was reduced from 14% to 5%, and the percentage of cells in G_2 was increased from 14% to 23%, suggesting that a transient G_2 arrest had occurred in at least a fraction of the cells; at this time point little or no sub-G_1 content cells were scored. By 2 days, however, 25% of the cells had a sub-G_1 DNA content and at 3 days after induction >60% of the cells had sub-G_1 DNA content indicating apoptotic death, with the remaining cells primarily arrested in G_1. Virtually all cells die after induction of p53 in p53-7 cells, thus, the arrest must be transient because the cells are not protected from eventually entering the apoptotic pathway. It should be mentioned that upon continued passage of p53-7 cells, the apoptotic phenotype of the cells was diminished and eventually lost. However, the levels of p53 were still high and the cells still exhibited the cell-cycle arrest component of the response.

When the second p53 cell line (p53-13) was analyzed, upon withdrawal of tetracycline, the amount of p53 within p53-13 cells was ~25–50% of that detected in p53-7 cells (Fig. 2A). Although p53-13 cells showed substantially slowed cell growth, there was in contrast with p53-7 cells, no reduction in cell number (Fig. 2B) nor did cells with sub-G_1 DNA content appear (Figure 2C). DNA histogram analysis showed that the percentage of S phase p53-13 cells was reduced from 31% to 15% within



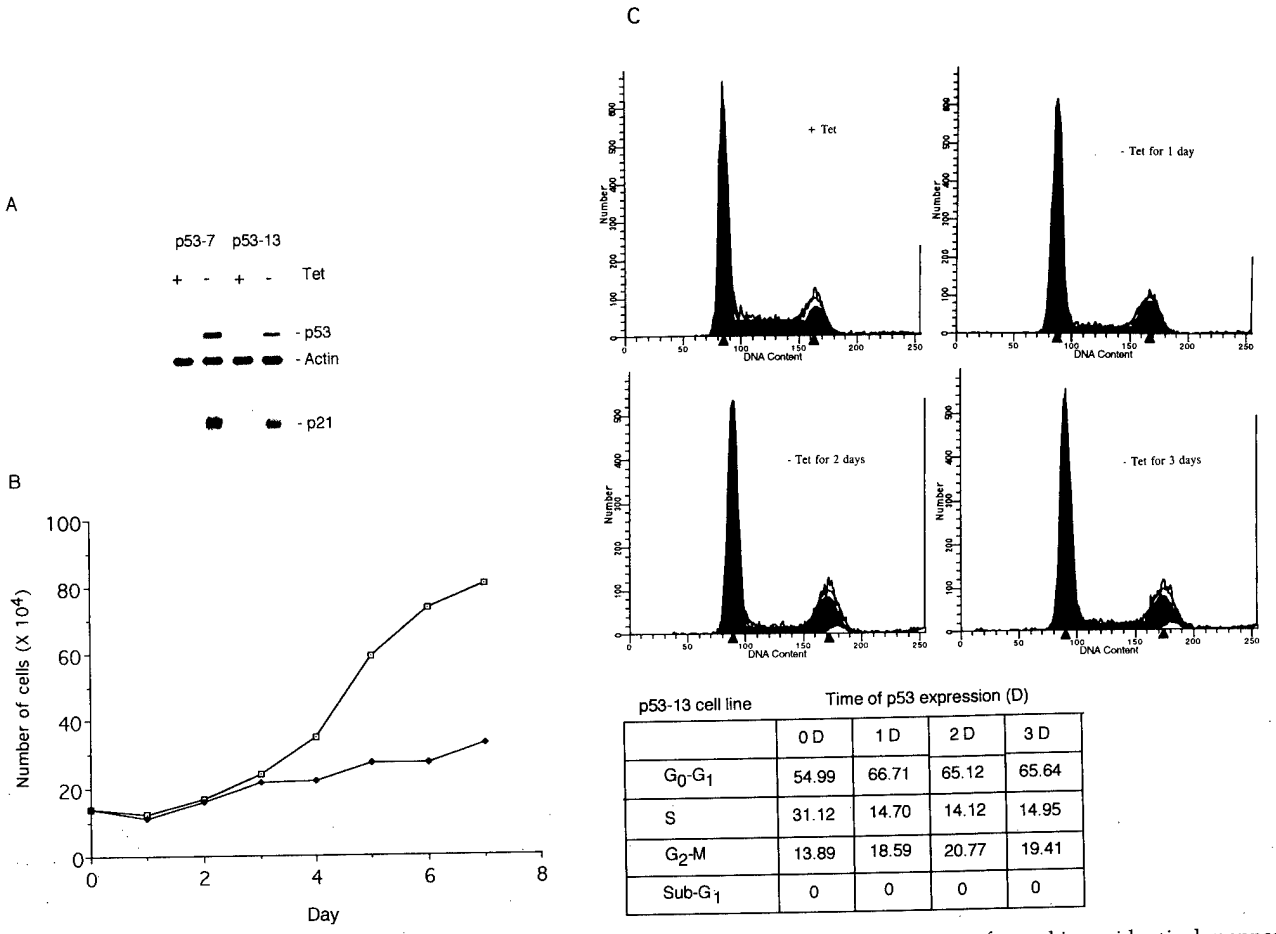
p53-7 cell line — Time of p53 expression (D)

	0 D	1 D	2 D	3 D
G_0-G_1	71.6	72.38	47.85	36.11
S	14.03	4.94	7.28	0
G_2-M	14.37	22.68	19.94	3.46
Sub-G_1	0	0	24.92	60.43

Figure 1. Induction of apoptosis by high levels of p53 in p53-7 cells. (*A*) Inducible expression of p53 and levels of actin in p53-7 cells in the presence or absence of tetracycline (1 μg/ml) for 24 hr, and in RKO cells treated with or without camptothecin (200 nM/ml) were assayed by Western blot analysis. The blot was probed with p53 monoclonal PAb1801 and actin polyclonal antibodies. (*B*) Growth rates of p53-7 cells in the presence (□) or absence (♦) of tetracycline were measured as described in Materials and Methods. Note that in this and other experiments there was a slight decrease in cell number at day 1 which is most likely due to trypsinization. (*C*) DNA contents were quantitated by propidium iodide staining of fixed cells at 0, 1, 2 and 3 days following withdrawal of tetracycline as described in Materials and Methods. (*Bottom*) Percentage of cells in the G_o-G_1, S, G_2-M, and sub-G_1 phases of cell cycle at 0, 1, 2, and 3 days following withdrawal of tetracycline was calculated from flow cytometric measurements of DNA content.

1 day following p53 induction and after 2 to 3 days the proportion of S phase cells remained at that low level (14%) (Fig. 2C). p53-13 cells, therefore, exhibit a significant arrest in both G_1 and G_2, but no apoptosis. The induction of p53 in both p53-7 and p53-13 cells, notably, was accompanied by a marked increase in the amount of detectable p21 (Fig. 2A).

Because the only obvious difference between the p53-7 and p53-13 cells was the amount of p53 detected after induction, we tested whether the level of p53 was, in fact, capable of regulating the apoptotic versus arrest response. This was done by varying the amounts of tetracycline in the culture medium of the high producer p53-7 cells. The results showed that low concentrations of tetracycline allowed for a partial expression of p53, whereas more complete withdrawl of tetracycline caused greater amounts of p53 to accumulate (Fig. 3A). Cells were counted at day 0, 1, or 3 following induction of p53 to different extents. The number of surviving cells was generally inversely proportional to the amount of p53 expressed and directly proportional to the amount of tetracycline present in the culture media (Fig. 3B). With the conditions resulting in the two lowest amounts of p53 induced (that is, 40 and 20 ng/ml of tetracycline), however, there was evidence of some increase in cell number, albeit significantly less than with no induction. Thus, intermediate levels of p53 caused slowed cell growth and cell arrest but not a significant amount of cell death, whereas levels close to or the same as those seen with full induction caused loss of cells comparable to that shown in Figure 1. We conclude from these data that the level of p53 in the p53-7 Saos2 cell line can determine whether the cells undergo growth arrest or apoptosis.



p53-13 cell line	Time of p53 expression (D)			
	0 D	1 D	2 D	3 D
G_0-G_1	54.99	66.71	65.12	65.64
S	31.12	14.70	14.12	14.95
G_2-M	13.89	18.59	20.77	19.41
Sub-G_1	0	0	0	0

Figure 2. Induction of cell-cycle arrest by low levels of p53 in p53-13 cells. The experiments were performed in an identical manner to those in Fig. 1.

DNA damage can sensitize cells to p53-mediated apoptosis without affecting the level of p53 protein

Effectors of DNA damage have been shown to increase the amount of p53 in cells by a post-transcriptional mechanism (Maltzman and Czyzyk 1984; Kastan et al. 1992; Lu and Lane 1993). Because the quantity of p53 induced was clearly a determinant of the switch between arrest and apoptosis in p53-7 cells, we wished to test whether the levels of p53 in the low producer cell line, p53-13, could be augmented after DNA damage, and whether the cells would now undergo apoptosis. Camptothecin (CPT), a topoisomerase inhibitor and cancer therapy drug, has been shown to induce DNA damage in cells (Nelson and Kastan 1994). Moreover, as shown in Figure 1A, we have confirmed that treament of RKO cells with CPT results in a significant induction of p53 protein levels. When p53-13 cells were treated with increasing amounts of CPT in the presence or absence of tetracycline we observed that even without p53 induction there was a modest apoptotic response to CPT, suggesting that Saos2 cells can undergo DNA damage-associated apoptosis in a p53-independent manner (Fig. 4B). Unexpectedly, however, when p53 was induced in CPT-treated p53-13 cells there was a significant increase in the number of apoptotic cells (Fig. 4B), and yet no discernable increase of p53 protein levels (Fig. 4A). Thus, p53 and CPT cooperate in Saos2 cells to cause a strong apoptotic response, and this occurs in a manner that is independent of p53 protein accumulation.

p21 induction in Saos2 cells leads to arrest but not apoptosis

The cyclin-dependent kinase inhibitor p21 (WAF1), a potential mediator of p53 tumor suppression, has been shown in a number of studies to be strongly induced by p53. Consistent with these results p21 expression was markedly increased in both p53-13 and p53-7 cells after removal of tetracycline (Fig. 2A). Because the low p53 producer cells (p53-13) did not undergo apoptosis this

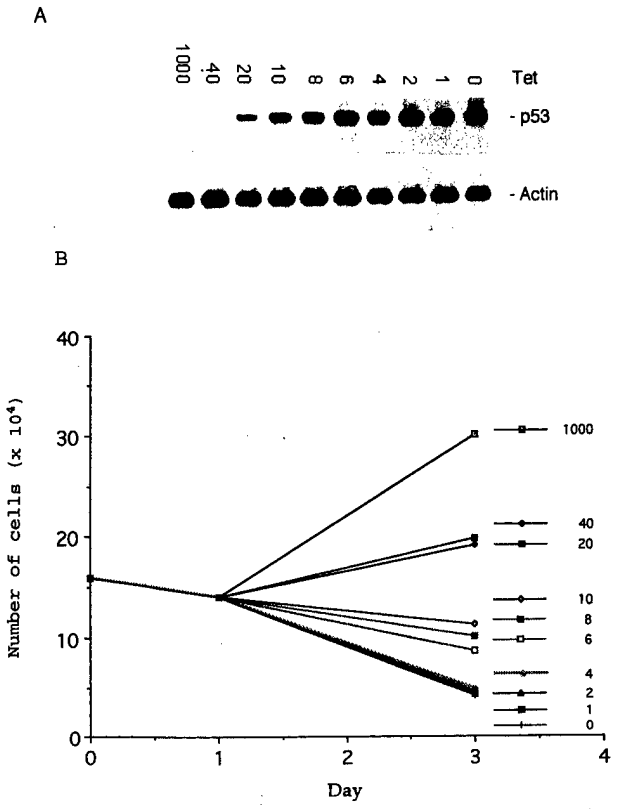


Figure 3. The level of p53 determines cell death or arrest. (*A*) Inducible expression of p53, and levels of actin in p53-7 cells in the presence of 1000, 40, 20, 10, 8, 6, 4, 2, 1, and 0 ng/ml of tetracycline as indicated were assayed by Western blot analysis. The blots were probed with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies. (*B*) The growth rates of p53-7 cells in the presence of varying concentrations of tetracycline were measured as described in Materials and Methods.

suggested that p21 induction alone is not sufficient for apoptosis. To directly analyze the role of p21 in cell death or arrest, we examined the cellular response to induction of p21 in the absence of p53. One of the p21-inducible cell lines (p21S4) expressed p21 protein to an even greater extent than in induced p53-7 cells (Fig. 5A). However, upon maximal p21 induction, p21S4 cells underwent cell-cycle arrest with no evidence of apoptosis (Fig. 5B). DNA histogram analysis showed that in the p21S4 line S-phase cells were reduced from 32% to 14% following 3 days of p21 induction and that cells were arrested in both G_1 and G_2 (Fig. 5C). It is notable that the extent of arrest at day 1 after induction of p53-7 cells was greater than that in p21 cells (cf. Fig. 2B and Fig. 5B), suggesting that p21 may not be the sole mediator of arrest after p53 induction. This observation is consistent with the results of Brugarolas et al. (1995) and Deng et al. (1995) indicating that the absence of p21 led to only a partial defect in G_1 arrest in response to radiation. Our data thus strongly suggest that p21 on its own is unable to produce an apoptotic response.

A weak and delayed apoptotic response is induced by a transactivation-deficient mutant form of p53

A p53 double mutant p53(gln22/ser23) was shown to be defective in transactivation (Lin et al. 1993) presumably because of the inability of this mutant to bind to TAFs that are critical for p53-mediated activation (Lu and Levine 1995; Thut et al. 1995). This mutant, however, has produced contradictory results as to whether transcriptional activation is necessary for p53-mediated apoptosis (Haupt et al. 1995; Sabbitini et al. 1995). To determine whether apoptosis can occur through a p53 transactivation-independent pathway in Saos2 cells, a cell line, 22/23-4, that expresses high levels of inducible transactivation deficient p53(gln22/ser23) was used. Consistent with evidence from in vitro studies (Lin et al. 1994) and transient transfections (Lin et al. 1995), this mutant form of p53 was transcriptionally inert because endogenous p21 was not induced even by high levels of

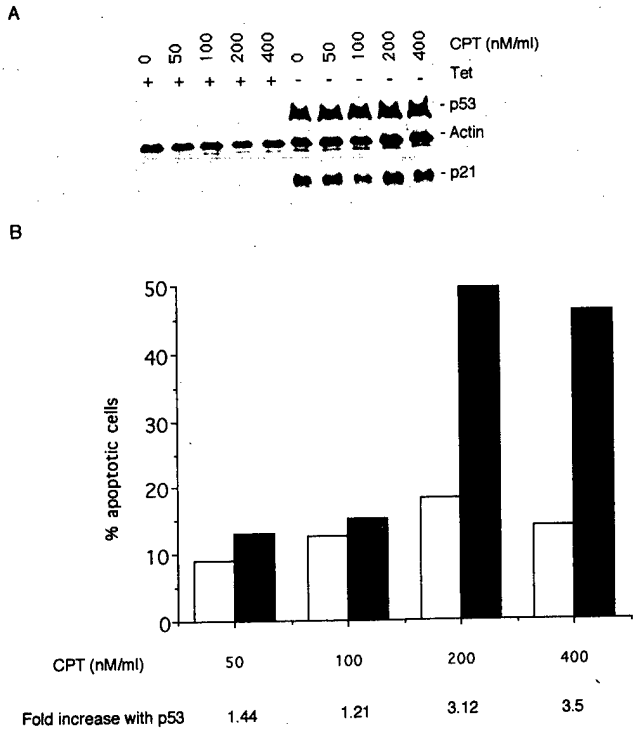
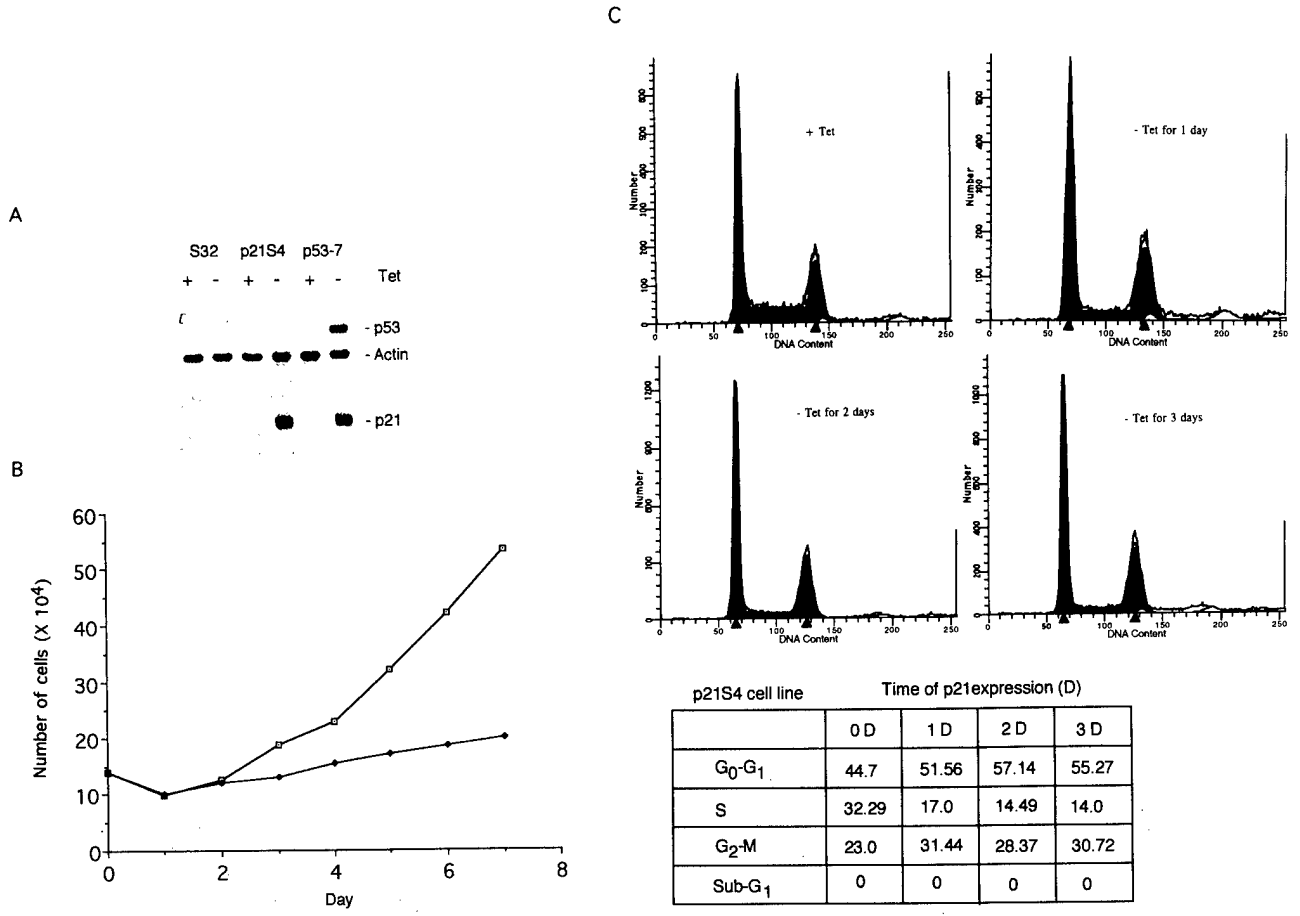


Figure 4. p53 sensitizes cells to undergo apoptosis by CPT-induced DNA damage. (*A*) Levels of p53, p21, and actin were assayed by Western blot analysis in p53-13 cells in the presence or absence of tetracycline for 24 hr, followed by treatment with CPT at concentrations of 0, 50, 100, 200, and 400 nM/ml for another 24 hr. (*B*) Number of apoptotic cells of the untreated or CPT-treated p53-13 cells in the presence (open bars) or absence (solid bars) of tetracycline.



p21S4 cell line — Time of p21expression (D)

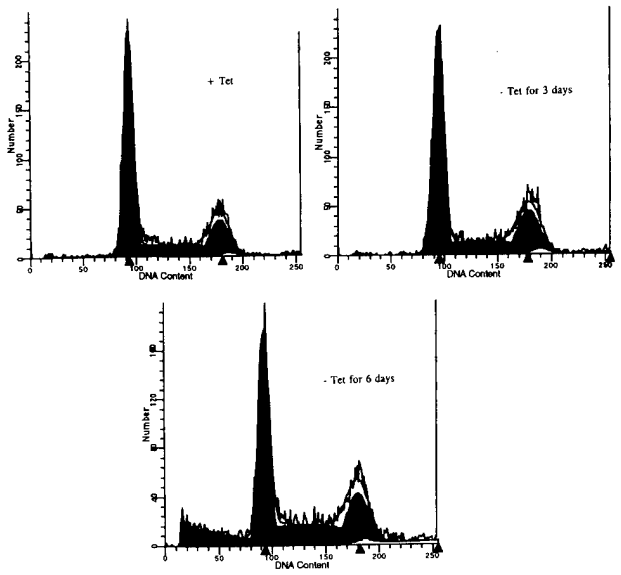
	0 D	1 D	2 D	3 D
G_0-G_1	44.7	51.56	57.14	55.27
S	32.29	17.0	14.49	14.0
G_2-M	23.0	31.44	28.37	30.72
Sub-G_1	0	0	0	0

Figure 5. p21 induction leads to cell cycle arrest but not apoptosis. The experiments were performed as those in Fig. 1.

p53(gln22/ser23) (data not shown). The amount of p53 in 22/23-4 cells was approximately equivalent to that detected in p53-7 cells. Consistent with the lack of induction of p21 in 22/23-4 cells, no detectable cell-cycle arrest was observed after induction of mutant p53 as observed by DNA histogram analysis (Fig. 6). Nevertheless, in these cells p53(gln22/ser23) reproducibly induced cell death (Fig. 6), although to a lesser extent and with delayed kinetics as compared with wild-type p53. Thus, those cells that did not undergo apoptosis contained a normal S-phase DNA content and presumably kept cycling. The apoptosis induced by p53(gln22/ser23), although reduced, was significantly greater than either the background levels of cell death that occur in the presence of tetracycline, or than in cells expressing mutant p53 completely defective in apoptosis (see Fig. 8, below, for comparison). Our data confirm and extend observations by Oren and colleagues (Haupt et al. 1995), who showed that apoptosis can be brought about by p53 mutants such as p53(gln22/ser23) that are defective in sequence-specific transactivation in transiently transfected HeLa cells. These data also provide clear evidence that the abilities of p53 to induce cell-cycle arrest and apoptosis are genetically separable.

The p53 carboxyl terminus is necessary for efficient apoptosis

p53 contains an autoinhibitory region within the last 30 amino acids of the protein. Deletion of this region generates a p53 protein that is activated for DNA binding in vitro (Hupp et al. 1992) and that is comparable to full-length p53 in activating transcription in transient transfection assays in cells (Halazonetis and Kandil 1993; L. Ko, unpubl.). To determine the cellular response to p53 lacking the carboxy-terminal 30 amino acids p53(ΔC30), cell lines expressing this p53 variant were isolated. One of these lines, p53(ΔC30)-6, contained at least twofold more p53 than the high producer cell line p53-7 when normalized to the cellular actin protein levels (Fig. 7A), although still in the range of p53 induced in DNA-damaged RKO cells. Consistent with observations that the ΔC30 mutant is functional in transactivation, p21 was induced in p53(ΔC30) cells to a similar extent as in wild-type p53 cells (Fig. 7B). Although the growth of p53(ΔC30)-6 cells was completely arrested upon induction, the cell number did not decrease detectably throughout the time course of the experiment (Fig. 7C). Because very slow but detectable cell growth had been



22/23-4 cell line Time of p53 expression (D)

	0 D	3 D	6 D
G_0-G_1	56.94	55.02	38.49
S	23.67	22.69	24.73
G_2-M	19.39	22.29	19.71
Sub-G_1	0	0	17.06

Figure 6. Induction of a delayed apoptosis but not arrest by p53 transactivation defective mutant p53 (gln22/ser23). DNA contents were quantitated by flow cytometric analysis in 22/23-4 cells at day 0, 3, and 6 following withdrawal of tetracycline. The percentage of cells in various phases of cell cycle at day 0, 3, and 6 after withdrawal of tetracycline.

observed for both the low p53 producer cell line p53-13 (Fig. 2B) and high p21 producer cell line p21S4 (Fig. 5B), and both cell lines do not undergo apoptosis, the flat growth curve of p53 (ΔC30)-6 cells suggested the possibility that a minor proportion of p53(ΔC30)-6 cells underwent apoptosis. Consistent with this idea, DNA histogram analysis of p53(ΔC30)-6 cells taken over 4 days showed that upon induction of p53(ΔC30), a weak apoptotic response with delayed kinetics ensued (Fig. 7D). Therefore, in this cell line and in others (data not shown), p53(ΔC30) can induce apoptosis, but far more weakly than full-length p53, suggesting that the carboxy terminal 30 amino acids of p53 are required for efficient apoptotic activity.

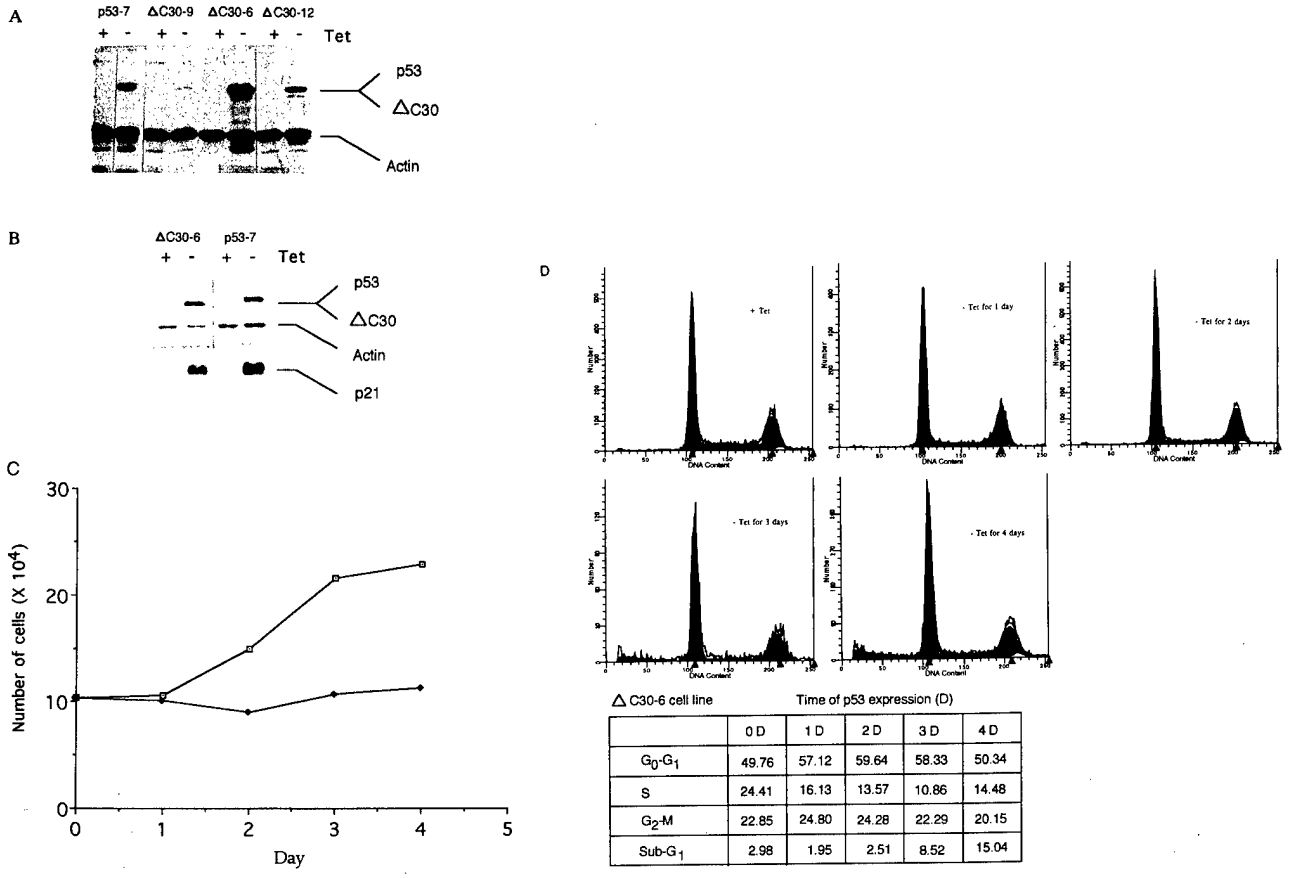
A full apoptotic response to p53 in tumor cells requires both sequence-specific transactivation and carboxy-terminal regulatory domains of p53

To gain further information as to whether the carboxy-domain of p53 would itself be sufficient to induce apoptosis in the absence of the amino-terminus, a Saos2 cell line p53(ΔN96)-5 expressing amino acids 97 to 393 [p53(ΔN96)] was used. p53(ΔN96)-5, which lacks the entire transactivation domain, is capable of sequence-specific DNA binding (L. Jayaraman and C. Prives, unpubl.) but is completely defective in transactivation (Pietenpol et al. 1994). Upon withdrawal of tetracycline, p53(ΔN96)-5 cells expressed markedly higher levels of truncated p53 than in the high producer wild-type p53 cell line, p53-7 (data not shown), yet induction of p53(ΔN96 had no measurable effect on cell growth or arrest (Table 1 and Fig. 8). This indicates that neither DNA binding nor the carboxy-terminal domain are sufficient for apoptosis.

Our results show that p53 alleles with mutations within the amino- and carboxyl termini were inefficient but not completely inert in inducing apoptosis in Saos2 cells. Tumor-derived mutant forms of p53 contain intact amino and carboxyl termini, but are incapable of binding specifically to p53 responsive elements and of transactivation (for review, see Gottlieb and Oren 1996; Ko and Prives 1996). To determine if such mutants would display any apoptotic activity in Saos2 cells, cell lines which contained inducible mutant forms of p53 [p53(ser249) or p53(his175)] were generated. Upon induction of high levels of either p53(ser249) or p53(his175), essentially no changes in growth or survival were detected in the Saos2 cells as compared with either the uninduced state or the parental cell from which they were derived (Table 1 and Fig. 8). These results confirm that tumor-derived mutant forms of p53 are inert for inducing the apoptotic response. Note that although we show here only data for hot-spot mutant p53 proteins p53(his175) and p53(ser249), we have observed that several additional tumor derived mutants are unable to induce apoptosis as well (Friedlander et al. 1996; data not shown).

H1299 cells with inducible wild-type and mutant forms of p53 confirm and extend results in Saos2 cells

During the course of the experiments described above with Saos2 cells we decided to use the same strategy to generate a number of additional inducible cell lines in another p53-null human cell background. We chose H1299 cells because, although like Saos2 osteosarcoma cells they are easily transfectable and can undergo p53-mediated apoptosis, they are of a different cellular origin (small cell lung carcinoma) and, importantly, in contrast to Saos2 cells, they express the RB tumor suppressor protein (data not shown). Because a relationship between RB and p53 has been established in many experimental models (for review, see White 1996) it was of interest to determine the response of these cells to the different forms of p53 that were tested in inducible Saos2 cells. The results of our experiments are summarized in Table 1. As was observed with Saos2 cells, one high p53 producer H1299 cell line underwent apoptosis after induction while a low producer H1299 cell line underwent



Δ C30-6 cell line	Time of p53 expression (D)				
	0 D	1 D	2 D	3 D	4 D
G_0-G_1	49.76	57.12	59.64	58.33	50.34
S	24.41	16.13	13.57	10.86	14.48
G_2-M	22.85	24.80	24.28	22.29	20.15
Sub-G_1	2.98	1.95	2.51	8.52	15.04

Figure 7. The carboxyl terminus of p53 is required for full apoptosis. (*A*) Inducible expression of wild-type p53 and p53(ΔC30), and levels of actin in p53-7, p53(ΔC30)-9, p53(ΔC30)-6, and p53(ΔC30)-12 cell lines in the presence or absence of tetracycline (1 μg/ml) for 24 hr. The blot was reacted with p53 monoclonal PAb1801 and actin polyclonal antibodies. (*B*) The inducible expression of wild-type p53, p53 (ΔC30) and p21, and levels of actin in p53-7 and p53(ΔC30)-6 cells in the presence or absence of tetracycline (1 μg/ml) for 24 hr. The blot was reacted with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies, and p21 monoclonal antibody, respectively. (*C*) and (*D*) The experiments were performed in an identical manner to those in Fig. 1B and 1C.

arrest. However, the high producer H1299 cells displayed a more rapid and extensive apoptotic response than seen with the Saos2 high producer cell line. The increased kinetics seen with this cell line may be caused by the faster doubling time of H1299 cells (e.g., 24 hr) as compared with Saos2 cells (~48 hr). Interestingly, a cell line with inducible p53 lacking the first 22 amino acids was as potent in inducing apoptosis as wild-type p53. Since p21 accumulated after induction in these cells, this suggests that p53(ΔN22) is transcriptionally active, although whether this truncated p53 is fully comparable to wild-type p53 is not yet established.

Consistent with results observed in Saos2 cells, H1299 lines expressing ser249, his275, or ΔN96 mutant forms of p53 were completely unable to induce apoptosis (Table 1). Moreover, cells expressing the carboxy-terminally truncated mutant p53(ΔC30) or the *trans*-activation-defective mutant p53(gln22/ser23) underwent apoptosis with reduced kinetics and extent over the time course examined. Importantly, a p53 variant that contained both the amino-terminal double mutation at residues 22 and 23 but that also lacked the carboxy-terminal 30 amino acids when expressed at high levels in H1299 cells, was completely inert in inducing apoptosis or growth arrest. This finding provided the strongest evidence that both the amino- and carboxyl terminus of p53 are required for apoptosis in tumor cells.

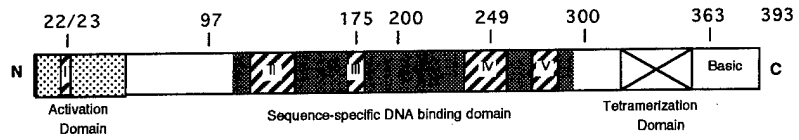
Discussion

Inducible cell lines provide insight into p53 responses in tumor cells

The cell lines described above have provided several novel observations about the cellular response to p53. We show for the first time that within a given clonal cell line the level of p53 can determine whether cells arrest or die. We also demonstrate that although DNA damage can cooperate with p53 to elicit an apoptotic response, this occurs without detectable alteration in the amount of the p53 protein. Furthermore, our results show that the arrest and apoptotic response are genetically separable activities of p53. Finally, our data suggest that the p53 protein has multiple domains that function in in-

Table 1. *Cell lines expressing inducible p53 or p21*

Protein expressed [a]	Saos-2 [b]	H1299 [b]	Apoptosis [c]	Arrest [c]	Cycling [c]
Wild-type p53 (H)	1	2	+++	+ [d]	-
Wild-type p53 (L)	1	5	-	+	-
p53 (ΔN22) (H)	nd	2	+++	+ [d]	-
p53 (ΔN22) (L)	nd	2	-	+	-
p53 (22/23) (H)	3	2	+	-	-
p53 (22/23) (L)	3	3	-	-	+
p53 (ΔC30) (H)	2	2	+	+	-
p53 (ΔC30) (L)	3	2	-	+	-
p53 (22/23ΔC30) (H)	nd	1	-	-	+
p53 (ΔN96) (H)	4	1	-	-	+
p53 (ser249) (H)	4	4	-	-	+
p53 (his175) (H)	4	3	-	-	+
p21 (H)	3	3	-	+	-



[a]Proteins were detected by Western blotting with p53 specific monoclonal antibodies PAb1801 or PAb421, or p21 specific monoclonal antibody AB-1 (Oncogene Science). Clones were divided where possible into high (H) and low (L) producers when protein levels differed by at least twofold.
[b]Number of individual clones of Saos-2 or H1299 cells expressing inducible p53 or p21.
[c]Apoptosis, arrest, or cycling states of cells were determined by growth curves and FACScan analysis.
[d]In cells that underwent massive apoptosis, cell arrest was transient.

ducing cell death and that these domains cooperate to produce a full apoptotic response.

The role of p53 as a transcriptional regulator in arrest and apoptosis

The response of Saos2 cells to the induction of p53 was dictated by the quantities of protein produced. When levels of p53 were lower, cells showed slowed or arrested growth, whereas at higher levels of p53, cell death ensued. As a transcriptional regulator, p53 binds to its cognate sites in p53 responsive genes and activates transcription of those genes. Cooperation between two separate p53 binding sites in the p21 promoter (El-Deiry et al. 1995) may allow for its activation by relatively little p53. Indeed, our preliminary results suggest that even with very low levels of p53 in p53-7 cells, p21 shows maximal activation (data not shown), and, thus, very lit-

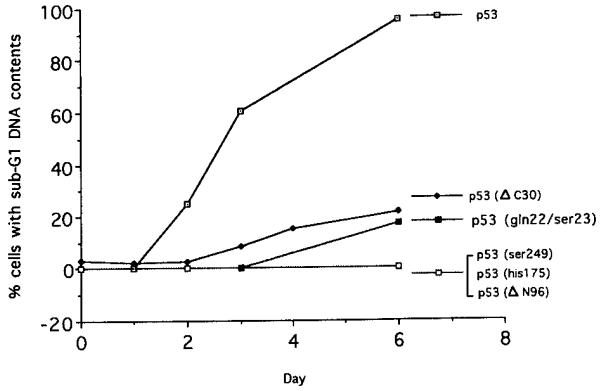


Figure 8. A full apoptotic response requires intact wild-type p53. Summary of the percentage of cells with sub-G_1 DNA contents that express inducible wild-type p53, p53(ΔC30), p53(gln22/ser23), p53(ΔN96), p53(his175), and p53(ser249) at various times following withdrawal of tetracycline.

tle p53 is required to drive expression of p21 and consequently to effect growth arrest.

It is likely that p53 transactivation also contributes to the apoptotic response because the cell death induced in response to the transactivation-defective p53 p53(gln22/ser23) occurs with delayed and reduced kinetics as compared with that seen with wild-type p53. p53(ΔC30), which lacks the carboxy-terminal 30-amino-acid regulatory domain but has comparable ability to activate transcription, also induces a weak and delayed apoptosis. However, the doubly altered mutant p53(gln22/ser23ΔC30) is inert for such activity (Table 1). Therefore, our data imply that although the ability of p53(gln22/ser23) to induce apoptosis is *trans*-activation-independent, that of p53(ΔC30) is *trans*-activation-dependent, highlighting the fact that p53 acts to induce apoptosis by at least two discrete pathways.

Because p21 induction is not correlated with p53-mediated apoptosis in the cell lines examined, there may be alternate p53 target genes involved in apoptosis that might be bound relatively weakly by p53 and would thus require more p53 protein to ensure sufficient site occupancy for transcriptional activation. Interestingly, the tumor-derived mutant forms p53(ala143) (Friedlander et al. 1996) and p53 (pro175) (Ludwig et al. 1996) are defective in inducing apoptosis but can induce transcription from a limited subset of p53 responsive elements. These mutants can activate transcription from promoters with responsive elements from p21, mdm-2, and cyclin G, but not Bax or IGFBP3 genes whose cognate sites, notably, are bound relatively poorly by p53. Although the Bax gene is an obvious candidate for an apoptotic p53 target gene, induction of Bax RNA or protein was not observed in Saos2 cells (data not shown), implying that other p53 responsive genes are activated in these cells. We are currently examining whether other candidate target genes such as IGFBP3 are activated by p53 in these inducible cell lines.

The role of p21 in arrest and apoptosis

Cell lines expressing p21 underwent arrest but not apoptosis in the absence of p53. The growth curves and FACS profiles of cells expressing p21 were similar to those expressing lower levels of p53 (cf. Figs. 2 and 5). In each case a dramatically reduced growth rate was accompanied by arrest in both G_1 and G_2. Although we cannot rule out that other targets of p53 may also be involved, these data imply that the arrest response of Saos2 and H1299 cells to moderate levels of p53 is caused primarily by induction of p21. It is also clear that p21 induction in these cells is insufficient to induce apoptosis. This conclusion is derived from the following results: (1) High levels of p21 expressed either with or without p53 did not cause apoptosis in Saos2 cells; (2) the p53(ΔC30) cell line, which shows a very reduced apoptotic response, is as effective as wild-type p53 in inducing p21 and cell cycle arrest; and (3) the transcriptionally defective p53 mutant p53(gln22/ser23) can not induce p21 (nor can it effect a cell-cycle arrest) and yet it can induce apoptosis, albeit to a lesser extent.

Despite the fact that p21 was induced to comparable levels in p21- and p53-inducible cell lines, the high producer p53-7 cells underwent both arrest and apoptosis. The fact that apoptosis was the eventual fate of virtually all p53-7 cells indicates that the p21-mediated arrest in these cells is not sufficient to protect the cells from the death response.

Speculation on a transcription-independent role for p53 in apoptosis

Given data from previous studies as well as the data presented here, it is clear that p53 can induce apoptosis in Saos2 and H1299 cells without transcriptional activation. Clearly, however, in some cases transcriptional activation is required (Sabbatini et al. 1995; Attardi et al. 1996). Although there is as yet a lack of full understanding of the reason for the differences in requirements noted, it can be speculated that species, cell type, and immortalization status differences may be involved. The magnitude of the apoptotic response varied dramatically with the p53 mutant that was induced (Fig. 8 and Table 1). Because the extent and kinetics of apoptosis induced by intact wild-type p53 are far greater than those by either of p53(ΔC30) or p53(gln22/ser23), we propose that transcription-dependent and -independent apoptotic pathways induced by these p53 variants, respectively, cooperate to induce a full apoptotic response. Cells expressing p53(ΔN96), which lacks the amino-terminal 96 amino acids but has an intact carboxyl terminus, can not induce apoptosis. Taken together with the results of the p53(ΔC30) mutant we conclude that both amino- and carboxyl termini must be intact to produce a strong p53 apoptotic response. Again, the fact that a mutant p53 with both a mutated amino terminus and a truncated carboxyl terminus [p53(gln22/ser23ΔC30)] is absolutely inert for both apoptosis and arrest in H1299 cells underscores this conclusion. Paradoxically, however, tumor-derived mutants that contain intact amino and carboxyl termini are also completely inert for inducing apoptosis. It is well established that the one feature common to the tumor-derived p53 mutations is a defect in sequence-specific p53 DNA binding. Therefore, our results suggest that p53 might need to be bound to cognate sites in DNA but not necessarily activating transcription for it to be in the correct conformation for its role in apoptosis. Alternatively, the identification of the cellular proteins 53BP1 and 53BP2 that can bind to the central core region of wild-type but not mutant p53 (Iwabuchi et al. 1994) provides the possibility that there may be cellular proteins rather than DNA with which this region of p53 must associate.

To explain our results, we propose the following model (Fig. 9): Interactions with a bi- or multicomponent factor would be required to associate with regions both at amino and carboxyl termini of p53, when it is bound to DNA, to cause apoptosis. Whereas the amino-termi-

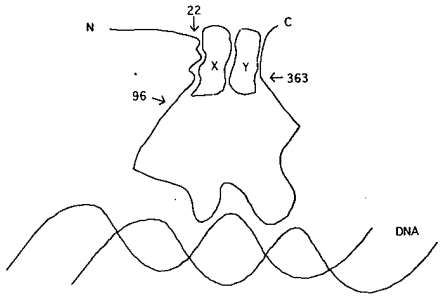


Figure 9. A model of p53-dependent apoptosis.

nal 22 amino acids of p53 would be dispensible for its interaction with the hypothetical factor at both ends [e.g., p53(ΔN22)], deletion of the entire amino terminus [e.g., p53(ΔN96)] would completely abrogate its interaction with the factor at the amino terminus, rendering this trucation of p53 incapable of inducing apoptosis. By contrast, mutations affecting part, but not all, of the amino- and carboxyl termini, such as p53(gln22/ser23) and p53ΔC30, would diminish but not completely destabilize interaction with the putative multiprotein complex, resulting in a lesser degree of apoptosis. Finally, amino- and carboxy-terminal double mutations p53 [e.g., p53(gln22/ser23ΔC30)] would completely disrupt complex formation so as to preclude an apoptotic response. In this model, massive over-expression of either the amino terminus alone or carboxyl terminus alone would be predicted to induce apoptosis to some extent, a suggestion that is borne out by results showing that overexpressed p53 amino-terminal fragment 1–214 (Haupt et al. 1995) or carboxy-terminal fragment 319–393 (Wang et al. 1996) can induce some degree of cell death.

A number of proteins have been reported to interact with p53 in vitro and in vivo. Of particular relevance to the function of p53 as a transcriptional regulator is its interaction with the basal transcription factors TFIID (Liu et al. 1993; Martin et al. 1993; Seto et al. 1992; Lu and Lane 1995; Thut at al. 1995) and TFIIH (Xiao et al. 1994; Wang et al. 1995), both of which contain more than one polypeptide component that interacts with p53. In each case these interactions involve both amino- and carboxyl termini of p53 (Xiao et al. 1994; Horikoshi et al. 1995; Lu and Levine 1995; Thut et al. 1995; Wang et al. 1995; Leveillard et al. 1996). Interaction of p53 with TFIID or TFIIH would most likely be facilitated when p53 is bound to DNA. Indeed, previously we reported cooperative interaction between p53 and TFIID when both were bound to DNA (Chen et al. 1993). It is also possible that other cellular factors might be involved because a considerable number of cellular proteins have been reported to interact with p53 (for review, see Ko and Prives 1996). Any of these and other as yet unidentified proteins may be involved in the initiation of the pathway toward apoptosis in response to p53.

p53 expressing cells are more sensitive to apoptosis when their DNA is damaged

Even though a low level of p53 is not sufficient to induce apoptosis in Saos2 cells, it can sensitize cells to undergo apoptosis following CPT-induced DNA damage. Because calcium phosphate-mediated DNA transfection of cells can induce p53 and an ensuing growth arrest (Renzing and Lane 1995), this procedure might also cooperate with expressed p53 to amplify the apoptotic response in transient transfection assays. This may explain why our experiments show a much weaker and delayed cell death for the p53(gln22/ser23) cell lines than previously reported when transient transfection assays were employed (Haupt et al. 1995). How these two agents, DNA damage and p53, cooperate is a matter of great interest. Additionally, it will be important to determine whether this cooperation occurs in cells other than Saos2. Although there is considerable evidence that p53 is stabilized after DNA damage, it has been speculated that DNA damage might also convert p53 to a more active DNA binding state (Lu and Lane 1994). This intriguing possibility is currently under investigation. We are excited by the potential of using low p53 producer cells to screen a variety of cancer therapy drugs that may cooperate with p53 to induce apoptosis. Thus, it is hoped that eventually more effective chemotherapeutic drugs can be identified.

Materials and methods

Plasmids

The pUHD15-1 neo plasmid contains the tTA transactivator gene and the neomycin gene as described in Resnitzky et al. (1994). Genes of interest were cloned into the 10-3 plasmid to allow their conditional expression as described in Gossen and Bujard (1992). The pBabe plasmid containing the puromycin resistance gene was used for secondary selection. To construct tetracycline regulated expression vectors, the following cDNA fragments were cloned into the 10-3 vector: wild-type p53, tumor-derived mutant forms of p53 (his175 and ser249), p53(gln22/ser23) (Lin et al. 1993), p53(ΔN22), p53(ΔN96), p53(ΔC30), p53(gln22/ser23ΔC30), or p21 (El-Deiry et al. 1993). To generate p53(gln22/ser23ΔC30), the carboxyl terminus of the p53(gln22/ser23) cDNA beginning at amino acid 144 at the *Pvu*II site was replaced by the carboxyl terminus of the p53(ΔC30) cDNA.

Cell lines and transfection, and selection procedures

The Saos2 and H1299 cells were purchased from the American Type Culture Collection. RKO cells were obtained from M.B. Kastan, Johns Hopkins University, Baltimore, MD (Kastan et al. 1992). All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Transfections were performed using the calcium chloride method (Chen and Okayama 1987). To generate cell lines expressing inducible proteins of interest, a two-step procedure was used (Resnitzky et al. 1994). First, low passage Saos2 or H1299 cells were transfected with pUHD15-1 neo and clones were selected and maintained in 400 and 250 μg/ml of G418 (Geneticin, Gibco), respectively. To test for cloned cell lines capable of inducing expression from the tetracycline promoter, the 10-3(p53) plasmid was transiently transfected in the presence of tetracycline (1 μg/ml) for 10–18 hr and the transfected cells were then split 1:2 and grown in the pres-

ence or absence of tetracycline. Cells were extracted 24–48 hr later, and expression of the p53 protein was determined by Western blot analysis. Two of the 15 clonal Saos2 cell lines (S32 and S2) and two of the 30 clonal H1299 cell lines (H15 and H24) were found to induce p53 expression upon withdrawal of tetracycline. Both S32 and H24 cell lines were used as parental cell lines for subsequent generation of inducible cell lines on the basis of their lower basal (leaky) expression of the tTA transactivation. Second, various 10-3 plasmids containing cDNAs encoding either wild-type or mutant forms of p53 or p21 were cotransfected with the puromycin selectable pBabe plasmid into either S32 or H24 cells. Clones were selected and maintained in the presence of 2 μg and 1 μg of puromycin (Sigma) per ml, respectively. Individual clones were screened for inducible expression of the p53 and p21 proteins by Western blot analysis using monoclonal antibodies against p53 and p21 as described below.

Immunoblot analysis

Cells were collected from plates in PBS, resuspended with 1× sample buffer, and boiled for 5 min. For immunoblot analysis, a standard procedure was followed as described previously (Chen et al. 1995. Monoclonal antibodies PAb1801 and PAb421 were used to detect p53. The affinity-purified monoclonal antibodies against p21 (Ab-1) and Bcl-2 (Ab-1) were purchased from Oncogene Science (Uniondale, NY) and affinity-purified monoclonal antibodies against Bax (P-19) and antiactin polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Sigma (St. Louis, MO), respectively.

Growth rate and cell-cycle analyses

To determine the rate of cell growth, 1×10^5 cells were seeded per 60-mm plate with or without tetracycline. The medium was replaced with fresh medium with or without tetracycline every 48 hr. At indicated times, cells were trypsinized and collected individually from at least two plates. Cells from each plate were counted with a hemacytometer at least twice. The average number of cells from at least two plates were used for growth rate determination.

For cell-cycle analysis, 2.5×10^5 cells were seeded per 90-mm plate with or without tetracycline. The medium was replaced every 48 hr as needed with fresh medium with or without tetracycline. At the indicated times, cell were trypsinized and fixed with 2 ml of 70% ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (PI) (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 hr. The percentage of cells in various cell cycle phases was determined by using the CellFit program.

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